Characterization of Influenza A-neutralizing, Heterosubtypic Antibodies Isolated with a Newly Developed Antigen

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Arkadiusz Maciej Wyrzucki

aus

Polen

Promotionskomitee

Prof. Dr. Alexandra Trkola (Vorsitz)

Prof. Dr. Lars Hangartner (Leitung der Dissertation)

Prof. Dr. Christian Münz

Zürich, 2013

“Man has gone out to explore other worlds and other civilizations without having explored his own labyrinth of dark passages and secret chambers, and without finding what lies behind doorways that he himself has sealed.”

Stanisław Lem, Solaris

Table of contents

1. Research summary

Seasonal Influenza A epidemics account for a higher morbidity than all other respiratory diseases taken together. Influenza viruses can tolerate changes in their antigenic structures, and are therefore capable of escaping pre-existing immunity. Such changes also result in a dramatic increase of viral resistance to available drugs. Furthermore, influenza vaccines protect only from one or few closely related strains, and therefore have to be annually formulated and applied. Thus, a novel, 'one shot' universal vaccine that provides immunity to wide range of Influenza A strains, including newly emerging strains, is highly desirable.

In my PhD thesis I aimed at defining universal epitopes of hemagglutinin (HA) – the major target protein for neutralizing antibodies on the Influenza A virus surface. It has been previously shown that some individuals possess cross-neutralizing antibodies that bind to more than one of the seventeen hemagglutinin subtypes. Crystal structures revealed that these antibodies interact with a hydrophobic groove in the HA stem region, and thereby block its pH-dependent conformational change necessary for viral-host membrane fusion. Most heterosubtypic monoclonal antibodies (hmAbs) only neutralized viruses from one of the two phylogenetic groups. However, two clones capable of neutralizing viruses from both phylogenetic groups have recently been described.

In this study I extended the knowledge about universal HA epitopes by selecting mAbs capable of binding and neutralizing Influenza A subtypes belonging to both groups. To this end, I built phage display Fab libraries based on the immune repertoire of a single individual, and these libraries were used in a modified selection process. I obtained a set of genetically distinct Fab clones presenting different HA cross-reactivity patterns. Two of the most promising candidates were further tested for virus-neutralization as IgG_1 molecules. Interestingly, one of these clones, referred to as mAb 1.12, neutralized a broad spectrum of Influenza A viruses belonging to both phylogenetic groups 1 and 2 (subtypes H1 to H15), whereas a second clone, mAb 3.1, neutralized several subtypes from phylogenetic group 1 (H1, H2, H5, H6). Together with our collaborators we solved the crystal structure of one of the complexes to define the recognized conserved HA epitope. Moreover, kinetic and mechanistic properties and antiviral mode of action was determined for both clones. I found that neutralization of these antibodies is virtually irreversible, that their antiviral activity is not impaired by apically binding, strain-specific serum antibodies and that they have to bind free virions, as their antiviral activity is dramatically reduced once the virus has bound to the cell surface.

In a preliminary approach I used the same antigen design that was used in the phage display since it was very efficient in selecting heterotypic antibodies. Surprisingly, two subsequent immunizations of mice with these antigens induced sera cross-reactive to all recombinantly expressed hemagglutinins (H1, H2, H3, H4, H5, H7, H12) as evaluated in ELISA.

In summary, with the results of this study I show that the development of a universal influenza vaccine directed against the conserved epitopes in the stem of the HA protein should be feasible and effective, even in the presence of pre-existing strain-specific serum antibodies.

2. Zusammenfassung

Die jährlichen Influenza A Epidemien sind für eine höhere Morbidität verantwortlich, als alle anderen respiratorischen Krankheiten zusammen. Das Influenzavirus ist besitzt die Fähigkeit, Änderungen in seiner antigenen Struktur tolerieren zu können und hat daher die Fähigkeit, bereits existierender Immunität zu entweichen. Ähnliche Änderungen führen auch zu drastisch erhöhter Resistenz gegenüber verwendeten antiviralen Medikamenten. Des weiteren schützen Influenzaimpfstoffe nur gegen einzelne oder wenige, eng verwandte Virenstämme und müssen deshalb jährlich neu generiert und angewendet werden. Ein Universalimpfstoff, dessen immunisierende Wirkung sowohl ein breiteres Spektrum, als auch neu aufkommende Virenstämme umfasst, wäre folglich erstrebenswert.

In dieser Arbeit, erstrebe ich die Erfoschung von Universalepitopen von Hemagglutinin (HA), dem Zielprotein für neutralisierende Antikörper auf der Oberfläche der Influenza A Viren. Es wurde bereits gezeigt, dass einzelne Individuen neutralisierende Antikörper besitzen, welche mehrere der siebzehn Hemagglutinin Subtypen binden können. Kristallstrukturen machen deutlich, dass diese Antikörper mit einer hydrophoben Furche der HA Stammregion interagieren und dessen pH-abhängige Konformationsänderung, welche nötig für die Membranfusion zwischen Virus und Wirtshzelle ist, blockieren. Die meisten mhAbs neutralisierten nur Viren, die zu einer der beiden phylogenetischen Gruppen gehörten. Kürzlich wurden jedoch zwei Klone beschrieben, welche die Fähigkeit besitzen, Viren von beiden phylogenetischen Gruppen zu neutralisieren.

In dieser Studie habe ich durch Selektieren von Fabs, welche Influenza A Subtypen aus beiden phylogenetischen Gruppen binden und neutralisieren können, das Wissen über die Universalepitope von HA erweitert. Um dies zu erreichen, generierte ich Phage-Display Fab-Bibliotheken basierend auf dem Immunrepertoire von einem einzelnen Individuum, welche dann in einem modifizierten Selektionsprozess weiterverwendet wurden. Dadurch erzielte ich einen Satz von genetisch unterschiedlichen Fab-Klonen, welche deutliche HA Kreuzreaktivität aufweisen. Zwei der meistversprechenden Kandidaten wurden des weiteren auf ihre neutralisierende Wirkung als IgG_1 Moleküle getestet. Interessanterweise konnte einer der Klone, mAb 1.12, die Neutralisation eines breiten Spektrums an Influenza A Viren beider Gruppen (Subtypen H1 bis H15) erzielen, während dem sich die neutralisierende Wirkung des zweiten Klons, mAb 3.1, auf mehrere Subtypen der Gruppe 1 beschränkte (H1, H2, H5, H6). Ermutigt durch diese Resultate, machte ich eine Kristallographie Analyse, um das hochkonservierte HA Epitope zu definieren, welches durch mAb 3.1. erkannt wird. Darüber hinaus charakterisierte ich kinetische Eigenschaften und die Wirkungsweise

der beiden Klone. Ich konnte zeigen, dass die Virus-Neutralisation durch heterosubtypische Antikörper praktisch irreversibel ist, dass vorbestehende stammspezifische Serumantikörper keinen negativen Einfluss auf diese Antikörper hat, aber auch dass heterosubtypische Antikörper freie Viren binden müssen, da sie einen grossen Teil ihrer antiviralen Wirkung einbüssen, nachdem das Virus an die Zelle angedockt hat.

In einer präliminären Studie verwendete ich das Antigen, das eine sehr hohe Effizienz bezüglich der Selektion von heterotypischen Antikörpern im Phage-Display bewiesen hat. Wie die Analyse mit ELISA zeigte, resultierten Mausexperimente mit zwei aufeinanderfolgenden Immunisierungen mit unserem Antigen in Seren, welche Kreuzreaktivität gegenüber allen getesteten rekombinant exprimierten Hemagglutininen (H1, H2, H3, H4, H5, H7, H12) zeigte.

Mit den Resultaten dieser Studie konnte ich zeigen, dass die Entwicklung eines Universalimpfstoffs, der die konservierten Epitope im Stamm vom Influenza HA angreift, möglich sein sollte und dass ein solcher Impfstoff auch bei vorbestehenden Stamm-spezifischen Serumantikörpern effektiv sein sollte.

3. Biology of Influenza A virus

3.1 Influenza A overview

Influenza viruses belong to the family *Orthomyxoviridae and are divided into 3 genera: A, B and C. Influenza A viruses* are classified into subtypes based on antigenicity of their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) [1]. Each Influenza A isolate is named according to the virus genus, name of host of origin (besides humans), geographic site of isolation, isolate number, year of isolation and HA and NA subtypes in brackets (e.g. A/California/7/2009 (H1N1)). New viruses traditionally were classified based on their cross-reactivity with monoclonal antibodies or polyclonal sera with specificity to a given subtype. Today, sequencing and phylogenetic analysis is used for the classification of influenza viruses. The first Influenza A virus, A/swine/Iowa/30 was isolated by Shope in 1930 followed by the isolation of the first human influenza virus by Smith, Andrews and Laidlaw in 1933 [1]. Since then 17 different HA and 10 different NA subtypes have been reported [2].

Influenza A viruses infect a wide variety of hosts. These include humans, horses, cats, birds, dogs, swine, whales and seals [1, 3]. Symptoms and severity of disease depend on species. For instance, in humans fatal infections are common whereas in aquatic birds, the natural reservoir of influenza A, infection is usually benign or even asymptomatic. The wide host-range of Influenza A can be attributed to several features of the virus. First, low requirements for host receptor recognition (see chapters 3.3 and 3.5.2.1 for details) [1, 4]. Second, the ability to accommodate extensive changes in surface protein composition in the processes of antigenic drift and shift [3-5].

Antigenic drift

Antigenic drift occurs when point mutations are being accumulated in HA and NA proteins leading to the emergence of new antigenic variants due to selection pressure exerted by neutralizing antibodies[1] (Figure 1). This process is a result of the lack of proof reading activity of the viral polymerase. It is estimated that in case of human viruses, such mutations are fixed at a frequency of less than 1% of amino acid composition per year [1]. This frequency is sufficient to enable the virus to constantly escape herd immunity, and to be maintained in circulation [1, 6]. Thus antigenic drift is the molecular mechanism responsible for seasonal Influenza A outbreaks as new viruses with slightly redecorated surface proteins are being generated each year.

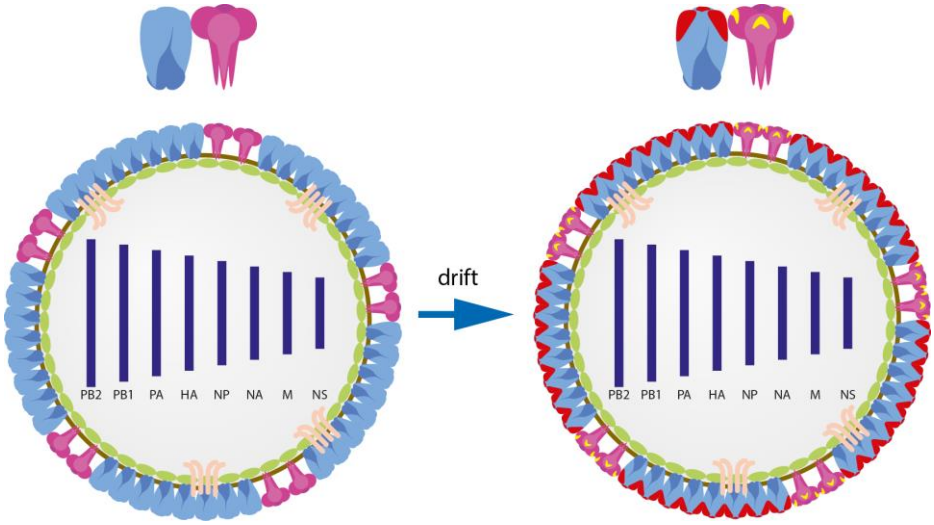


Figure 1. Antigenic drift of Influenza A virus. The two surface glycoproteins, HA and NA, being the subject of antigenic drift are depicted in blue and magenta, respectively. Mutations accumulated during the drift process are shown in red (HA) and yellow (NA). Adapted from Lars Hangartner.

Antigenic shift

Antigenic shift is a process in which a new HA or NA subtype is being introduced into human viruses leading to the emergence of a virus variant that is immunologically distinct from previously circulating strains [1, 6] (Figure 2). Since there is usually no preexisting immunity to such viruses in human population antigenically shifted viruses often cause Influenza A pandemics. A good example for antigenic shift is the emergence of pandemic viruses in 1918, 1957, 1968 and 1977 (see chapter 3.4 for details). Antigenic shift usually occurs when gene segments encoding for HA or NA proteins (sometimes with other viral gene segments) are being introduced into the background of human viruses. Such reassortment of the genetic information can occur when a cell or organism is simultaneously infected with both human and avian viruses, and the resulting virus is a mosaic between these two. Reassortment is suspected to primarily occur in swine as they can be infected with both avian and human isolates [1]. The new virus can be further transmitted between pigs and humans. An alternative way for antigenic shift to occur is direct transmission of avian or swine strain to humans, followed by adaptation of the virus to the human host. Such zoonotic cases have been well documented in the last 50 years [1, 7, 8].

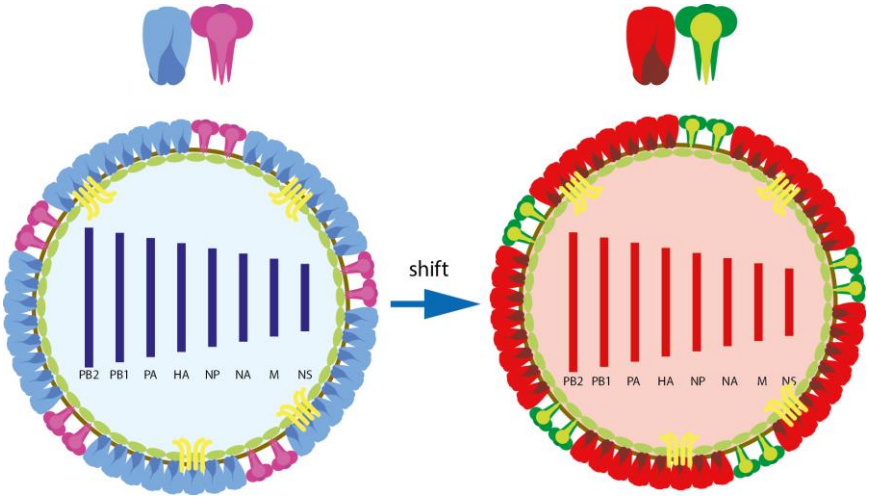


Figure 2. Antigenic shift of Influenza A virus. The exchange of HA (blue to red) and NA (magenta to green) between virus subtypes is depicted. Adapted from Lars Hangartner.

3.2 Structure of the virion

In electron microscopy, Influenza A virions are present in two forms: spherical with approximately 100 nm in diameter and filamentous with a length of 300 nm or more [1, 5] (Figure 3). The outer layer of both consist of host- derived membrane into which two types of glycoproteins, trimeric HA and tetrameric NA, and the M2 ion channel are inserted. HA spikes are abundant on the virus surface exceeding the number of NA molecules 4 fold, and the number of M2 channels 10-100 fold [5, 9]. Below the membrane, the viral matrix enclosing the core is formed by the M1 protein. Influenza A core consists of the nuclear export protein NEP (also known as the non-structural protein 2, NS2) and the ribonucleoprotein complex (RNP). RNPs contain viral genomic RNA and heterotrimeric RNA-dependent RNA polymerase. Each polymerase molecule consists of two ‘polymerase basic’ (PB1, PB2) and one ‘polymerase acidic’ subunits (PA) [5, 10]. Table 1 briefly summarizes the structural components of Influenza A virion indicating their function.

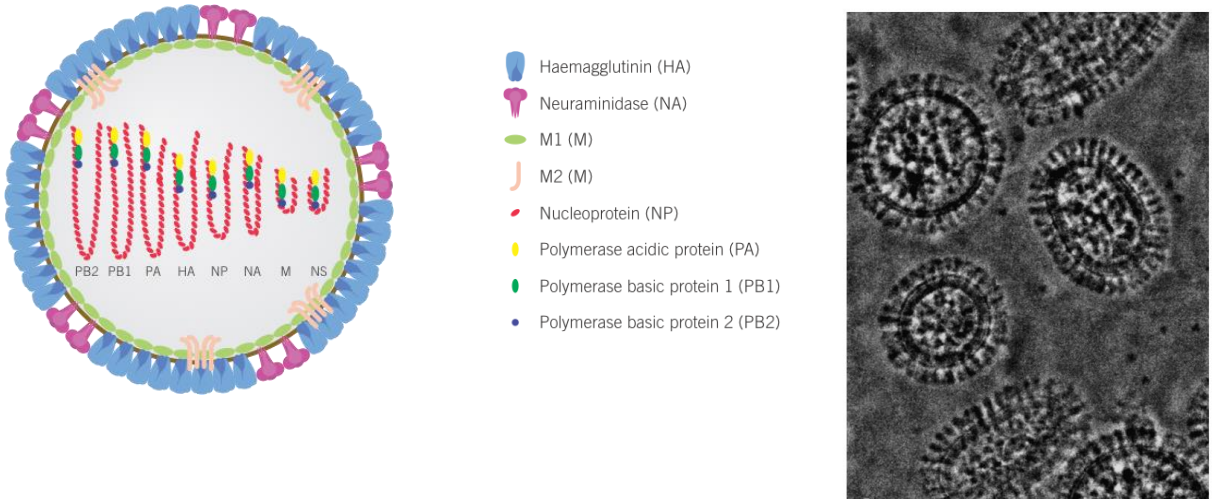


Figure 3. Structure of Influenza A virion. Schematic representation of Influenza A with virion components depicted (left, adapted from Lars Hangartner). Zernike phase contrast electron micrograph of ice-embedded influenza A viruses (right, modified from [11]).

The genome of influenza viruses consists of 8 segments of negative-sense, single-stranded viral RNA [1, 5]. Segments 1,3,4,5,6 encode only one protein (PB2, PA, HA, NP, NA respectively). Segment 2 usually encodes only the PB1 polymerase subunit, however some strains also express the accessory protein PB1-F2 from this segment using an alternate reading frame [12]. M1 and M2 proteins are both expressed from segment 7 with the latter one arising from alternative splicing [13]. Segment 8 encodes the interferon antagonist protein NS1 and, by mRNA splicing, also the NEP/NS2 protein. Each of the 8 segments ends with noncoding regions and has a helical hairpin made of highly conserved sequence on one side of the genome [1, 5, 14]. These conserved RNA stretches serve as recognition sites for the viral RNA-dependent RNA polymerase. Noncoding vRNA ends contain also the mRNA polyadenylation signal and packaging signals necessary for virus assembly.

protein	function	segment encoding
PB2	polymerase subunit, cap recognition	1
PB1	polymerase subunit, endonuclease activity, elongation	2
PB1-F2 (optional)	pro apoptotic	2
PA	polymerase subunit	3
HA	receptor binding, membrane fusion	4
NP	RNA binding and synthesis	5
NA	neuraminidase activity, virus release	6
M1	matrix protein, interaction with surface glycoproteins and vRNPs	7
M2	acidification of viral lumen, particle disassembly	7
NS1	IFN antagonist	8
NEP/NS2	nuclear export of vRNPs	8

Table 1. Components of Influenza A virion. For each viral protein a short description of its function and the number of encoding segment is given.

3.3 Influenza A 'life' cycle

Attachment to host cell surface

Influenza A virus uses its surface glycoprotein HA to bind sialic acid residues on host glycoproteins [4](Figure 11 in chapter 3.7). This quite unspecific binding enables infection of many different cell types and species, and is contributing to the wide spread of the virus around the globe. Hemagglutinins from different influenza A strains have preferences for the linkage of the sialic acid residues. Avian strains prefer cells with α2,3 linkage between sialic acid and galactose in the sugar backbone [5, 15]. This type of linkage is abundant in avian gut epithelia and therefore Influenza A is an enteric virus in birds. Human strains have a preference for α2,6 linkage which is predominant in the

upper respiratory tract. However, epithelial cells in the lower respiratory tract also contain the α2,3 linkage which means that humans can be infected with avian strains [16]. Due to limited accessibility of the lower respiratory tract for virus particles (comparing with upper respiratory tract) this type of infection is less likely but also more dangerous. Human infections with avian influenza strains are usually associated with severe pneumonia and fatality rate over 60% [17]. Details about the molecular recognition of host cell receptors are provided in chapter 3.5.2.1.

Virus entry and release into cytoplasm

Once the virus particle is attached to cell surface, it gets internalized into endosomes by endocytosis. Following acidification of endosome HA molecules undergo structural rearrangement leading to fusion of the viral and vesicle membrane[1, 4, 5]. In parallel, the M2 ion channel pumps protons inside the virion which leads to acidification of the viral lumen, disruption of viral protein- protein interactions and finally particle disassembly. The virus RNPs are then released into cytoplasm[18].

Viral RNA synthesis

RNPs are further transported into cell nucleus through the host's importin α/β pathway that recognizes nuclear localization signals on RNPs [5, 19]. Once arrived in the nucleus, the RNA-dependent RNA polymerase (RdRp) synthesizes two types of transcripts: mRNA and antigenomic RNAs that are complementary, positive-sensed copies of the genomic RNA (cRNA). The mRNA is exported from the nucleus and translated in the cytoplasm into viral proteins. Synthesis of viral mRNA is unique in this term that the polyA tail is transcribed directly from a stretch of uracil residues present at the 5' end of each RNA segment. Furthermore, the mRNA receives its cap structure from host mRNA molecules in a process called 'cap snatching' [10]. Briefly, the viral RdRp simultaneously binds viral and cellular mRNA molecules. Binding to host mRNA is most possibly mediated by the PB2 subunit of RdRp. Once both mRNA molecules are associated with RdRp the PB1 subunit cleaves the capped 5' end of the host mRNA using its endonuclease activity. This capped 10-13nt long RNA is then used as a primer for viral mRNA synthesis by PB1 [1, 20]. In contrast the antigenomic RNA serves as a template for the production of negative-stranded vRNA progeny [1, 5] .

Nuclear export and virus assembly

Viral mRNA is exported from nucleus into cytoplasm by the same machinery used for host cell mRNA [5]. It becomes associated with ribosomes at the ER and viral protein expression begins [21]. Following successful translation, properly folded HA, NA and M2 proteins contain apical sorting signals which direct them through the Golgi to the plasma membrane where they await newly synthetized RNPs. RNPs are formed in the nucleus by associating vRNA with viral proteins. It is believed that the lately expressed M1 protein redirects vRNPs used for transcription and replication earlier out of the nucleus [22]. M1 mediates the interaction between vRNA and NP as well as with NEP. The latter one is the driving force for translocation of RNPs from nucleus into cytoplasm. Once in the cytoplasm RNPs are brought in contact with the surface proteins HA, NA and M2- an interaction probably mediated by M1.

Virus release

It is assumed that accumulation of M1 protein on the cytoplasmic side of host membrane initiates virus budding [1, 5]. vRNPs present at the same site are being incorporated into the budding virus. This process seems to be regulated by packaging signals on the vRNA segments that ensure formation of complete particles [23]. Following budding, progeny virus particles stay attached to cell surface due to interaction of HA with sialylated glycoproteins [4, 5]. This interaction is abolished by the enzymatic activity of NA cleaving off sialic acid residues on both host and viral glycoproteins. This sialidase activity of NA not only releases virus particles from cell surface but also prevents them from aggregation with each other.

3.4 Epidemiology and economical impact

Seasonal flu

Each year Influenza A and B viruses infect hundreds of millions of people worldwide. Even though virulence of circulating strains is far lower than for pandemic flu, approximately half a million individuals succumb to influenza infections every year [1, 24]. There are currently two subtypes of Influenza A viruses circulating in human population: H1N1 and H3N2. The first subtype has most possibly accidentally been reintroduced into circulation in 1977 whereas the latter emerged during pandemic in 1968 (see below) [1]. Both subtypes are subject to antigenic drift, which means that each year new variants appear that have evaded the preexisting herd immunity sufficiently to cause a

seasonal epidemic. In the northern hemisphere, the flu season usually peaks between January and April, whereas in the southern hemisphere, outbreaks occur from May to September [25, 26]. This seasonality of Influenza A infections is believed to be associated with the low relative indoor humidity that may prolong the half-life of virus particles [1]. However this hypothesis is not in agreement with some experiments performed under controlled conditions as well with the fact that in tropical areas, like Southeast Asia, epidemics strike during rainy season.

Seasonal influenza mostly affects children age 2 and below, the elderly and people with underlying conditions [26]. The incidence of symptomatic influenza infections requiring hospitalization in the first 2 groups is around 10 times higher than for people age 1 to 44 (1 in 270 vs 1 in 2900, respectively) [1, 27]. In general, annual flu affects 10 to 20% of population however this number can reach 50% in case of a pandemic. The most common symptoms of infection include fever, nose discharge, fatigue, depression, diarrhea, body aches and sore throat [1, 25]. In severe cases, which are mostly caused by H3N2 viruses, pneumonia and secondary bacterial infections may lead to systemic failure and death with highest morbidity reported in elderly.

Influenza pandemics

As mentioned above, antigenic drift attributes to the emergence of new variants of circulating strains each year. However, it is the antigenic shift that is responsible for introduction of completely new virus types into the human population to which there is usually no preexisting immunity [1, 25, 26]. First convincing reports about influenza pandemics reach back to 16th century when in year 1580 an outbreak started in Russia and spread to Africa and Europe [28]. However, based on historical data, it is believed that Influenza A was causing local epidemics already in ancient civilizations [29]. First reliable epidemiological reports were collected between 1889 and 1892 when H2N2 (or H3N8) viruses caused a pandemic [25]. Soon after, the H2N2 (or H3N8) subtype was replaced by H3N2. Furthermore, H1N1 subtype was introduced into human population possibly in 1908 and was cocirculating with the aforementioned H3N2. The epidemiological situation was steady until 1918 when the most severe Influenza A outbreak took place. The so called ‘Spanish flu’ killed 20 million people worldwide within a year [25, 26]. However, this is believed to be an underestimate and the real number may have reached 50 million casualties. The pandemics started most possibly in Fort Funston in Kansas with a single soldier falling sick after having contact with pigs [1]. In the first of 3 phases, H1N1 spread rapidly around the globe. Although highly infectious it was causing mild symptoms similar to seasonal flu and affecting mostly young children and elderly. This changed dramatically in August 1918 when a more virulent form emerged that started the second pandemic wave. A sudden increase in morbidity and mortality was observed among young people aged 15 to 35, a phe-

nomenon that is still not completely understood. In general the 'Spanish flu' had a very high mortality rate reaching 2,5% (comparing to 0.1% in seasonal Influenza) with more than 99% of deaths affecting individuals younger than 65 (comparing to 10% for seasonal virus) [1, 25]. During second and third wave, patients were showing severe but typical influenza symptoms that in many cases developed into tracheobronchitis or secondary bacterial pneumonia leading to death. Some individuals have been identified post mortem with hemorrhagic lungs further confirming extreme virulence of the pandemic strain [25]. This severe virulence have been attributed later to the hemagglutinin protein and, to a lesser extend, to viral polymerase [30, 31]. Another factor contributing to high morbidity and mortality was the poor health condition of individuals after World War I. 'Spanish influenza' outbreak ended in 1919 but drifting variants of the H1N1 virus were circulating for the next 40 years. In 1957, viruses of the H2N2 subtype emerged in Southern China causing the 'Asian influenza' pandemic and completely replacing the H1N1 virus (Figure 4). Within a year the H2N2, a reassortant between human and avian viruses, caused 1 million deaths. In 1968, an H3N2 subtype containing avian hemagglutinin caused the 'Hong Kong' pandemics with an impact comparable to "Asian influenza'. The H3N2 virus spread rapidly around Asia, Australia and USA via military personnel returning from Vietnam. Luckily, the severity of this outbreak was modest most possibly due to preexisting immunity to N2 protein in human population [1]. From 1977 to present, the H3N2 subtype is co-circulating together with H1N1-bearing viruses that have probably been accidentally released from research facility in Russia or China, and that have caused a mild pandemic that was affecting individuals mostly in USSR and China with morbidity almost entirely limited to people age 25 or less.

Since the 'Russian flu' in 1977 two fatal Influenza A pandemics were feared. In 1997 avian H5N1 virus spread in poultry in Hong Kong infecting in parallel individuals working or visiting animal markets [32]. Although the infection was rapidly contained and no human-to-human transmission occurred, this was the first time an avian virus was reported to cause such lethal infection in humans. Since then variants of H5N1 virus circulate in poultry, sporadically infecting people with a mortality rate reaching 60% [1]. The second high risk event took place in 2009 when a new H1N1 strain, a multiple reassortant between human, avian and swine viruses, emerged in Mexico killing approximately 18 000 people worldwide [33]. Although at that time WHO called this outbreak a pandemic it is believed that this particular virus was not a serious threat to the human population.

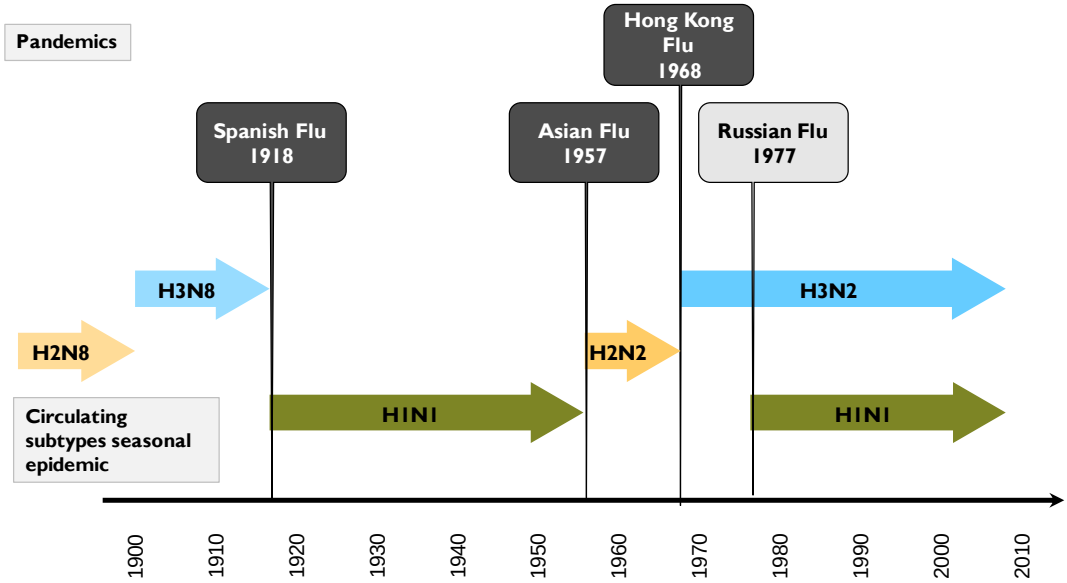


Figure 4. Chronological representation of Influenza A pandemics and epidemics. The upper part of the graph depicts influenza pandemics highlighted in grey boxes. Each pandemic introduces a new subtype into circulation as shown. Lower part indicates seasonal virus subtypes that were circulating in human population in the past. Adapted from Lars Hangartner.

Influenza in animals

Influenza A is capable of infecting many species including birds, dogs, cats, swine, horses, whales and seals [1]. Of major importance from the economical point of view are outbreaks in poultry. One of the most extreme cases was the aforementioned outbreak in 1997 in Hong Kong [34]. The highly pathogenic H5N1 strain showed 70-100% mortality rate. In total 1.5 million domestic birds have been either killed by the virus or culled. Due to rapid intervention, the spread of the virus was temporarily contained until 2001 when H5N1 first reappeared in Hong Kong causing later a severe outbreak in 2003 in Vietnam [1, 35, 36]. Up till now 100 million of domestic birds have been killed due to this outbreak around the world and the virus continues to cause substantial damage in poultry especially in Egypt and Indonesia. H5 and H7 avian subtypes have been reported to cause significant loss in poultry since 1950s [1]. The most dramatic outbreaks took place in Pennsylvania (H5N2, 1983, 17 million birds killed), Pakistan (H7N3, 1995, 3.2 million birds killed) and Netherlands (H7N7, 2003, 30 million birds killed) [1, 34].

Economical impact

The wide spread of Influenza A virus both in humans and animals has a significant economic impact each year. Several studies showed that financial loss due to seasonal flu is counted in billions of dollars in the US alone. A recent analysis performed by Molinari and colleagues in 2007 pointed out that the annual influenza epidemics (both A and B) account for 3.1 million hospitalized days, 31.4 million outpatient visits and a medical cost around $10.4 billion [37]. However, it has to be taken into account that the cost of medical treatment is only a small percentage of the total bill. The combined economic loss (work and school absence, changes in customer consumption patterns, loss related to deaths etc) related to seasonal flu was evaluated in this study for $87.1 billion (data for US population in 2003). This seems to be in agreement with the recent estimates of US$ 71-167 billion made by WHO [38].

Although seasonal influenza epidemics seem to be very costly, their economic burden would be very minor comparing to a real pandemic. According to the estimates of World Bank from 2008, a pandemic with similar death rates as during Spanish influenza would possibly cause a global GDP decrease by 4.8% (3 trillion US$) [39, 40]. Even a mild outbreak like the Asian flu is predicted to shrink the GDP by 2%. Similar studies have been performed for US only suggesting, that a pandemic affecting 15-35% of population would cost between $71.3 billion and $166.5 billion (as for 1999) [41].

What has to be kept in mind is the economic burden related to avian influenza outbreaks in poultry. H5 and H7 subtypes have been infecting poultry since a long time leading to millions of killed or culled birds [1]. A good example is the outbreak of H7N3 subtype from 2004 in British Columbia, Canada. Approximately 17 million animals have been killed leading to loss of CAN$ 380 million [42]. Noteworthy, this number is only part of a total cost of such outbreak. Even more severe economic impact in such cases is related to changes in consumer habits, decreased poultry import and loss of trust.

3.5 Hemagglutinin- the major surface antigen of Influenza A virus

3.5.1 Structure

Influenza A hemagglutinin (HA) is a type one membrane protein comprising approximately 550 amino acids [43]. It contains a transmembrane domain near the C-terminus and short cytoplasmic tail. HA consists of 2 subunits, HA1 and HA2, which are generated by proteolitic cleavage of the HA0 precursor (see below) and which are linked with a disulphide bond. In its mature form, HA is a homo-

trimer of dimers of 220 kDA and 135 Å in length. The dimer contains two domains: a membrane-distal globular head and a membrane-proximal elongated stem (Figure 5). The globular head is composed of HA1 residues 52 to 275 (H3 numbering) that mainly form β-sheets. The stem consists of the C- and N-terminus of HA1 (residues 11 to 51 and 276 to 329) and HA2. It forms a characteristic triple coil-coiled structure of α-helices. Each of the HA dimers has a receptor binding site located in head portion and fusion peptide, necessary for virus entry, at the N terminus of HA2 in stem.

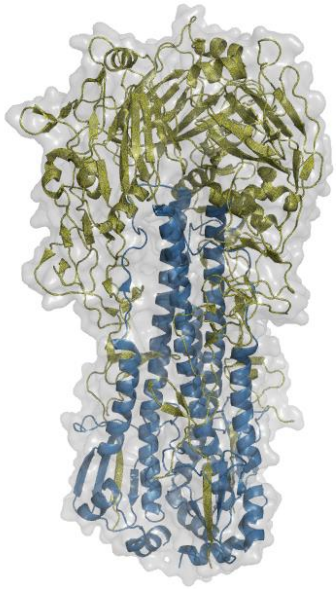


Figure 5. Structure of Influenza A hemagglutinin. Ribbon representation of HA1 (dark yellow) and HA2 (blue) subunits of hemagglutinin with protein surface depicted in transparent grey (prepared with PyMOL software, DeLano Scientific LLC, PDB entry 1MQL).

3.5.1.1 HA processing- from HA0 precursor to mature glycoprotein

HA is encoded by segment 4 of Influenza A genomic RNA [5]. Once the segment is transcribed, the mRNA is exported into cytoplasm and associated with ribosomes that drive protein synthesis. HA is expressed as HA0- a monomeric precursor protein [1, 43]. Folding of HA0 starts already during translation in parallel with the high mannose glycosylation [21]. Sugar residues are recognized by calnexin, a chaperon protein which supports further folding steps. Approximately 5 minutes after polypeptide chain termination, all intramolecular disulphides are formed and the HA0 monomer becomes DTT resistant. At this step the globular head is properly folded whereas stem region is still immature. Correctly folded homotrimers are assembled 5-10 minutes later in the ER or the ER-Golgi intermediate compartment. HA is then brought to cell membrane and incorporated into virus particles [5].

HA proteins require three major modifications to mature and become fully functional [4]. First, glycosylation is necessary to acquire a proper fold [21, 44]. It has been shown that complete deglycosylation leads to missfolded HA as the protein can not interact with the folding chaperon calnexin. Moreover, glycosylation is essential to overcome immune response [4]. Sugar residues on HA are synthesized by cellular machinery, and are therefore recognized as 'self'. This helps the virus to prevent formation of antiviral antibodies, or impairs binding of pre-existing antibodies. Second modification is palmitoylation of the three Cys residues present in the transmembrane region (one Cys) and cytoplasmic tail (two Cys) [1]. Mutation of those residues in a H1 virus led to loss of infectivity, whereas in case of a H3 virus, the impact on infectivity was moderate [45, 46]. The last modification is a proteolitic cleavage of the HA0 precursor into the HA1 and HA2 subunits. Cleavage releases the fusion peptide crucial for virus entry into host cell [4, 43]. In some of HA subtypes, the cleavage site Q/E-X-R is most likely recognized by tryptase Clara produced by Clara cells in the bronchiolar epithelium. This type of cleavage occurs either on the cell surface or on released virus particles. In case of some avian strains from the H5 and H7 subtype, cleavage occurs intracellularly [4, 47]. These strains contain a so-called polybasic region with a R-X-R/K-R motif that is recognized and cleaved by subtilisin-like enzymes such as furin and PC6.

3.5.1.2 Antigenic sites

The antigenic sites on HA have been extensively studied using x-ray crystallography, antibody-selected mutants and sequence analysis. All sites are located in the globular head and have been shown to share two features [1]. First, they mostly have a loop-like structure and are extensively glycosylated. Second, they can accept amino acid substitution without affecting HA fold and function. There are 5 antigenic sites identified: H3 HAs contain site A made of a loop formed by aa residues 140-146, site B containing a loop (aa 155-160) and α-helix (aa 188-198), site C formed by antiparallel sheet at the base of head portion, site D located close to trimeric interface and site E situated between C and A on the side of the head. Similar sites have been identified for H1 (Ca1, Ca2, Cb, Sa, Sb), and H2. Antigenic sites were shown to accumulate point mutations leading to the emergence of antigenic variants. A good example is the human H3 subtype circulating since 1968. Between 1968 and 2013 residues 142 through 146 and 155 through 160 have all been substituted, some of them multiple times [4].

3.5.1.3 Hemagglutinin subtypes

There are currently 17 HA subtypes known. Sixteen of these subtypes are divided into 2 phylogenetic groups that contain several clades [48, 49] (Figure 6). New subtypes are described over time,

e.g. in 1980 only 12 subtypes have been reported [50]. All subtypes besides H17 are maintained in aquatic birds, the primary reservoir for Influenza A viruses. H17 has been discovered in fruit bats in 2012. Each species has a set of subtypes by which it is preferentially infected. H1, H2 and H3 are found in humans, H3 and H7 infect equines and H1, H2, H3, H5 and H9 circulate in swine [1, 25, 26, 34]. Significant differences in amino acid composition are observed within each subtype and even more between each other. For instance, past and present human H3 isolates may differ in up to 20% whereas subtypes can only share as little as 30% of aa sequence [1, 4]. Surprisingly, despite this sequence variation hemagglutinins maintain proper fold and function. This phenomenon might be attributed to the fact that mutations in HA mostly accumulate in the globular head which seems to be tolerant to change. In contrast, the stem portion containing the sophisticated membrane fusion machinery, is relatively conserved [48, 51].

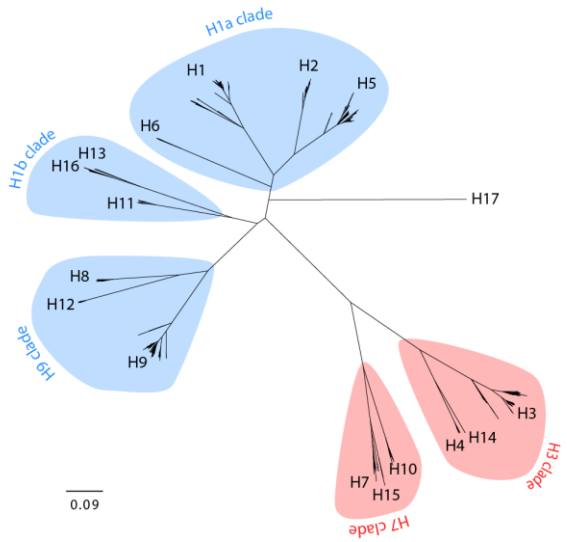


Figure 6. Phylogeny of Influenza A virus hemagglutinin. Group 1 (blue) and group 2 (red) are shown including clade division and subtypes (H1 to H17). Figure was prepared based on the alignments of 6592 recent HA protein sequences from the NIH Flu database. Adapted from Lars Hangartner.

3.5.2 Function

3.5.2.1 Receptor binding and specificity

Hemagglutinin binds sialic acid on host glycoproteins with affinities in low millimolar range [4]. Binding is mediated by hydrophobic interactions and hydrogen bonds [47]. Each monomer of Influenza A hemagglutinin has a receptor binding site that contains a set of conserved amino acids (Tyr98, Trp153, His183, Tyr195) at its tip, and three conserved structural elements, the 130- and 220-loops and the 190-α-helix at the edges [4, 47]. Mutational study showed that residues at positions 98 and 183 are absolutely essential for receptor recognition, whereas substitution of amino acids at positions 153 and 195 may only partially inhibit receptor binding. It is believed that due to very low affini-

ty multiple HA molecules have to be involved in interaction with host receptors to allow efficient virus attachment to cell surface [47].

Although sialic acid is present on various glycoproteins in many species, different HAs show a clear receptor binding specificity. Avian and equine viruses bind sialic acid with α2,3-linkage to galactose, human viruses prefer a α2,6-linkage and swine viruses interact with both [1]. Structural studies revealed that in case of avian HA the sugar is bound in an extended conformation whereas human viruses HAs bind the glycan in a bent conformation [43, 47]. Mutation experiments showed that only few residues are responsible for maintaining receptor specificity e.g. amino acid substitutions Q226L and G228S change the H5 preference from avian to human type of sialic acid linkage. Recent data suggest that similar mutations occurred in case of the Spanish flu H1N1 strain where H1 of avian origin was adapted to recognize human receptors and caused a worldwide pandemic [1].

3.5.2.2 Membrane fusion and virus entry

A second important function of HA is mediating the membrane fusion. The main structural element involved in this process is the fusion peptide, comprising the first 10 N-terminal amino acids of HA2 subunit [4]. After proteolitic cleavage of the HA0 precursor the released fusion peptide is hidden in a negatively charged cavity in the HA stem. Once the virus particle is internalized into endosome, and the pH in endosomal compartment drops below 6 (values between 5 and 6 have been described), the HA protein undergoes extensive structural rearrangements [5, 52] (Figure 7). Thereby, the globular head is displaced aside, and the flexible loop between A and CD-helix in the coiled-coil stem structure acquires an α-helical conformation and pulls the aforementioned A-helix along. The fusion peptide leaves the stem's cavity as it is being pulled up by the A-helix. This structural rearrangement displaces the fusion peptide by over 100 Å from its initial location, and results in its insertion into the endosomal membrane[1]. In this manner, a bridge between viral and endosomal membrane is established. Parallel insertion of fusion peptides from several HA molecules finally opens a pore in both membranes, and the vRNP particles are released into cytoplasm[5]. Noteworthy, fusion can occur at higher pH in case of specific HA mutants. It has been shown that viruses selected in presence of amantadine (weak base increasing the endosomal pH) can fuse membranes at neutral pH [4, 53].

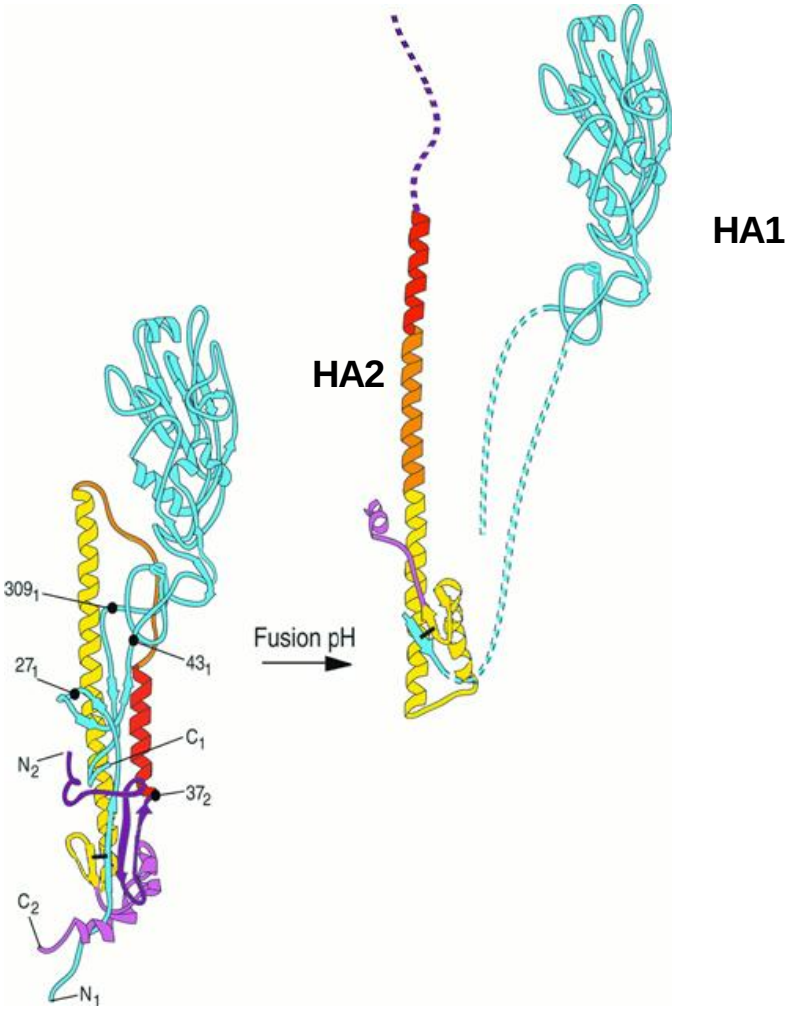


Figure 7. Structural rearrangement of hemagglutinin at low endosomal pH. Upon acidification of endosomal compartment the loop (orange) connecting A (red) and CD (yellow) helix in HA2 subunit adopts a helical conformation and pulls up the fusion peptide (purple) towards endosomal membrane. In parallel the HA1 subunit is dislocated to enable membrane fusion. Modified from [4]

3.5.3 Antigenic properties

3.5.3.1 Nature of antibody response to hemagglutinins

Antibodies to HA, NA, NP, M1 and M2 proteins can be detected following infection with Influenza A viruses [54]. Among these, the humoral responses to HA and NA have proven to clear the virus and to provide protection from re-infection[1]. Multiple studies have shown that the level of serum antibodies to HA and NA correlates with restriction of virus replication in the respiratory tract [55]. Antibodies to HA neutralize the infectivity of Influenza A by several different mechanisms (see chapter 3.6 for details).When passively transferred in mice, they can provide protection even in the

absence of B or T cells [1, 56]. This observation further stresses the crucial role of humoral immunity in clearing Influenza A viruses. During the course of infection, three isotypes of antibodies specific for HA are produced: IgA, IgG, IgM. All three classes are present on mucosal surface with the IgA and IgM isotypes being the most abundant. Of these, the secreted IgA has been shown to be sufficient to prevent infection and to provide resistance to antigenically similar strains for 3 to 5 months after initial infection [57].

The antibody response to HA has a limited duration time and usually only protects against similar Influenza A strains [1]. Two factors contribute to this. First, the serum IgG and mucosal surface IgA levels significantly decrease during the first year after the initial infection. Second, new influenza A viruses with redecorated antigenic sites emerge every year or every few years (Figure 8). Such viruses are usually not recognized by antibodies elicited by the previous form of the virus [4, 51]. For a long time it has been believed that the repertoire of serum antibodies to HA is very limited. Indeed the vast majority of HA specific antibodies bind to the highly variable globular head, and are relatively easily escaped by the virus. Nevertheless, it has recently been shown that following infection or vaccination, antibodies binding the conserved HA stem are elicited in humans [58-61]. Corti and colleagues proved that these stem-reactive mAbs are present at a very low concentration in human sera of many individuals and that their production is further boosted by vaccination. Although these antibodies can neutralize Influenza A, at the concentrations they are present in serum neutralization of virus is not possible. Moreover, the same sera can neutralize pseudotype viruses (with a much lower density of HA distribution on surface) emphasizing difficulties in accessibility of the stem epitope on full virus particle.

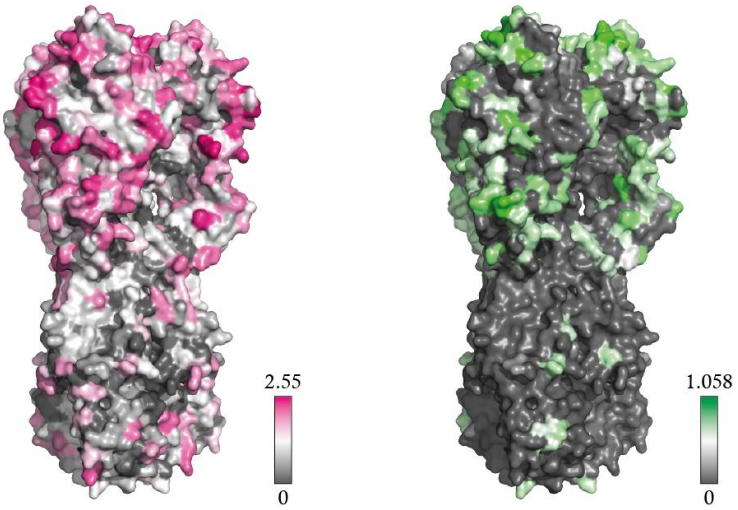


Figure 8. Variability of Influenza A hemagglutinin. Changes in amino acid composition between subtypes (left) and within the H1 subtype (right) have been depicted in gradient colors using the Shannon's entropy algorithm. Residues that are not changing or are changing only rarely are shown in grey whereas residues being the subject of frequent mutations are shown in magenta or green. Figure was prepared based on alignments of 6592 recent HA protein sequences from the NIH Flu database (left) and based on the alignments of 8219 non-identical H1 sequences from the NIH Flu database (right) using PyMOL software (DeLano Scientific LLC). Adapted from Lars Hangartner.

3.6 Antibodies binding to Influenza A hemagglutinin

3.6.1 Subtype specific antibodies

Following Influenza A infection or vaccination, the majority of produced antibodies is strain specific. Some of these antibodies may show cross-reactivity to homosubtypic strains, i.e. belonging to the same subtype. The very limited breadth of these antibodies, however, arises from the fact that they usually bind to epitopes on the genetically highly variable hemagglutinin head [51]. Strain- and subtype-specific mAbs have been widely used in the past to describe the antigenic sites of various Influenza A isolates arising from antigenic drift (either natural or selected *in vitro*). For instance, Gerhard and colleagues selected a set of A/Puerto Rico/8/1934 (H1N1) antigenic variants by passaging the parental strain in presence of various monoclonal antibodies. They have further analyzed these variants by testing their binding to 58 anti-HA mouse mAbs [62]. They have found that viruses clus-

tered into 4 groups corresponding to 4 distinct antigenic sites on the H1 molecule (see chapter 3.5.1.2 for details). In 1982 testing several other escape mutants with a set of 32 additional murine mAbs has discovered the 5th antigenic site [63]. Similar studies have been performed to describe the antigenic properties of H3 hemagglutinin and later also for H2 HA where a 6th unique antigenic site has been revealed in the stem region [64-66].

Recently a set of human antibodies with broad cross-reactivity within the subtype has been also described. In 2009 Kubota-Koketsu and colleagues reported several mAbs isolated from human PBMCs using hybridoma technology [59]. Some of these antibodies, like clones B-1 and D-1, showed binding to several antigenically distinct H3N2 viruses. B-1 and D-1 epitopes have been mapped at the side of HA globular head- a portion that seems to be conserved to some extend in the H3 subtype. Interestingly, although potently neutralizing, those mAbs showed very poor hemagglutination inhibition (HI) suggesting other mechanisms for virus neutralization rather than blocking the receptor-binding site. Whittle et al have described another interesting human monoclonal antibody, termed CH65, in 2011 [67]. This mAb has been shown to neutralize 30 out of 36 tested H1N1 strains. This broad cross-reactivity results from the binding of CH65 to the relatively conserved HA receptor-binding pocket. The antibody inserts its CDRH3 loop into the pocket mimicking to some extended the binding with sialylated receptor.

3.6.2 Heterosubtypic antibodies

3.6.2.1 Monoclonal antibodies cross-reactive to HAs from phylogenetic group 1

Identifying antibodies capable of neutralizing several HA subtypes has long been a goal in the field of Influenza A studies. In 1993 the first such heterosubtypic monoclonal antibody C179 has been described [68]. It was isolated from mice that have been repetitively immunized with A/Okuda/1957 (H2N2) virus. C179 showed a broad cross-reactivity, and neutralized viruses from the H1, H2, H5 and H9 subtypes [69]. However, little binding without neutralization has been reported for H6 isolates. Noteworthy, the antibody did not bind any virus from phylogenetic group 2 HAs. In several animal studies, C179 protected to various degree mice that were lethally challenged with H1, H2 and H5 viruses [70]. Initial epitope mapping using escape variants showed that residues at position 318 in HA1 and 52 in HA2 are absolutely crucial for C179 binding, and indicated the existence of a highly conserved epitope in the stem of the HA protein. This epitope was distinct from the previously described epitopes in the antigenic sites present on the globular head. Recently, the crystal structure of C179 in complex with HA from A/Japan/305/1957 (H2N2) has been solved, confirming the interaction of the antibody with HA stem [71] (Figure 9). It is now known that C179 utilizes the heavy and light

chains to bind residues on both the HA1 and HA2 subunits. In the heavy chain HCDR1 and HCDR3 loops are involved in the recognition of a hydrophobic groove in proximity of the fusion peptide whereas Trp34 in LCDR1 has been shown to interact with Lys38 in HA2.

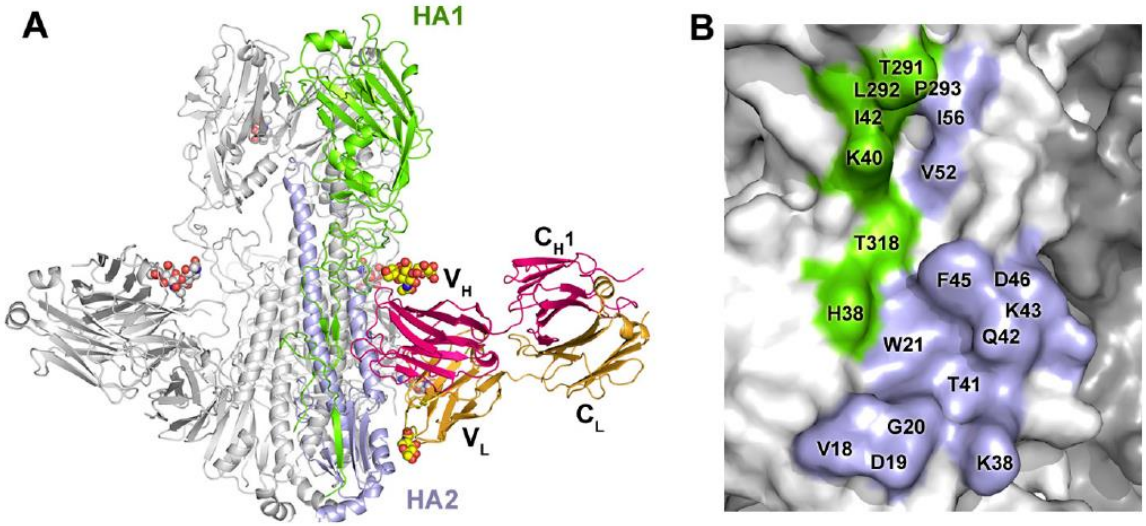


Figure 9. Epitope recognized by mAb C179. (A) Crystal structure of Jap57/H2-Fab c179 complex. One HA/Fab protomer of the trimeric complex is colored with HA1 in green, HA2 in light blue, Fab heavy chain in magenta, Fab light chain in orange. N-linked glycans shown as spheres are colored by atom type. The two remaining protomers are colored in gray. (B) Residues in HA1 (green) and HA2 (violet) recognized by Fab c179 (modified from [71]).

In 2009, two groups reported almost in parallel the discovery of a new class of broadly cross-reactive human, monoclonal antibodies recognizing the HA stem [48, 51]. mAbs CR6261 and F10 were selected independently from human phage display libraries. CR6261 and F10 were able to neutralize majority of viruses from phylogenetic group 1, and protected mice from lethal challenge with H1 (CR6261, F10) and H5 (CR6261). However, no interaction with group 2 HAs has been detected. Like C179, neither of these mAbs did inhibit hemagglutination but rather neutralized the virus by preventing the structural rearrangement of HA at low pH. However, both of the newly discovered antibodies used exclusively their heavy chains to contact HA which is different from C179. The involvement of a single chain in epitope recognition is quite common for clones selected from phage display libraries and is believed to be an artifact of the selection process. The crystal structures of CR6261 in complex with H1 or H5 HA revealed that this antibody uses mainly HCDR1 and HCDR2 loops to bind the antigen while the involvement of HCDR3 is limited. This feature is pretty uncommon as in majority of antibodies crystallized to date a long HCDR3 contributes most to the binding.

Also, the structure of F10 in complex with H5 showed an involvement of all 3 HCDRs. Moreover, besides interacting with HA stem residues, HCDR3 also stabilized the conformation of HCDR2. Furthermore FR3 of F10 has been found to support the binding of HCDR1 and HCDR2. In case of both mAbs the recognized epitope contains stem-associated HA1 residues and elements of HA2 (fusion peptide, A-helix). Interestingly, the heavy chain of CR6261 and F10 evolved from the same V_H 1-69 germline family. A characteristic feature of VH 1-69 is the presence of Met/Iso/Leu/Val at position 54 and Phe residue at position 55 [72]. These hydrophobic residues at the tip of HCDR2 are crucial for recognition of hydrophobic patches on HA stem. Similar interaction involving V_H 1-69 HCDR2 has been previously reported for broadly cross-reactive mAbs binding other proteins (e.g. HIV gp120) [73, 74].

There are 2 subtle structural differences in the CR6261 and F10 epitope between group 1 and 2 HAs [48, 51, 75] (Figure 10). These differences are believed to cause the lack of binding of these 2 mAbs to group 2 viruses. First, position 38_1 (subscript 1 denotes HA1 and 2 denotes HA2 subunits) in four out of 6 members of group 2 HAs is occupied by Asn (His in group 1) and glycosylated. The presence of this sugar was predicted to decrease the binding of CR6261 and F10. Indeed, CR6261 binding to H5 mutant glycosylated at position 38_1 was significantly reduced. Second, the $Trp21_2$ residue is differently oriented in group 1 and 2. As this residue is recognized by Phe at the tip of HCDR2 in both CR6261 and F10 and significantly contributes to binding, the different orientation is believed to completely abolish the interaction of these 2 mAbs with group 2 viruses.

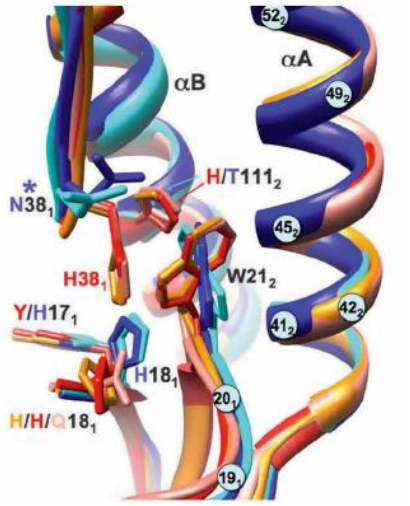


Figure 10. Structural differences between group 1 and group 2 hemagglutinins in the epitope recognized by mAb F10. An overlay of H1, H5 and H9 (group 1, shaded red and yellow, PDB 1RU7, 2IBX and 1JSD) and H3 and H7 (group 2, shaded in blue, PDB 1MQL and 1TI8). Residues interacting with mAb F10 were depicted including the two crucial positions 38_1 and 21_2 (subscripts 1 and 2 denote residues in HA1 and HA2 subunit, respectively). Modified from [48].

In 2010 another group 1 cross-reactive antibody was discovered. mAb PN-SIA49 has been initially described as neutralizing multiple H1N1 isolates [76]. Later, the same antibody has been proven to neutralize all group 1 subtypes besides H9 and to provide protection from lethal challenge with H1N1 and H5N1 viruses [77]. Interestingly, the heavy chain of PN-SIA49 belongs to germline family V_H 3-23 suggesting a different binding mode compared to CR6261 and F10. Competition for HA binding

with C179 and alanine scanning studies mapped the PN-SIA49 epitope in HA stem, further supporting the notion that this part of hemagglutinin bears highly conserved structures.

3.6.2.2 Group 2 cross-reactive monoclonal antibodies

One antibody with cross-reactivity limited to hemagglutinins from phylogenetic group 2 has been reported to date [78]. mAb CR8020 was isolated from a human donor using hybridoma technology. This antibody binds to most group 2 HAs (H3, H7, H10, H15) with affinities ranging from ~1 to 35 nM with the exception of H4 and H14 subtypes that were bound with K_D ~500 and ~1000 nM, respectively. Similarly, CR8020 was found to neutralize various H3, H7 and H10 isolates with IC_{50} values ranging from 1.7 to 13.1 µg/ml. However, it failed to neutralize H4-subtyped virus. Furthermore, CR8020 protected mice from A/Hong Kong/1/1968 (H3N2) or A/Chicken/Netherlands/621557/2003 (H7N7) infection in both prophylactic and therapeutic setups. Crystal structure of CR8020 in complex with H3 revealed that the mAb uses its both chains to bind a stem epitope in a close proximity to the viral membrane, and thus much more membrane proximally than the epitopes described for CR6261 and F10. The binding area was almost entirely composed of residues of the HA2 subunit residues with the exception of residue 325 in HA1. As expected, CR8020 neutralized virus particles by preventing the structural rearrangement of HA at the pH of membrane fusion- a mechanism previously described for other HA stem reactive antibodies [48, 51, 75, 79]. A detailed comparison showed that the epitopes of CR8020 and CR6261 are distinct with only 2 residues ($Asp19_2$ and $Leu38_2$) shared between them.

Although the CR8020 epitope is relatively conserved in group 1 HAs, no binding to group 1 hemagglutinins have been observed. Two structural differences may account for this observation [78]. First, Gln or Thr residues are present in group 2 at the HA2 position 34 which is occupied by bulkier Tyr in group 1. Structural simulations showed that such Tyr would possibly cause steric hindrance with HCDR3. Indeed, binding to an H3 Gln34Arg mutant was reduced over 100 fold, indicating the importance of position 34 in CR8020 epitope recognition. Second, a conserved carbohydrate is present at $Asn21_1$ in group 1. This sugar is believed to clash with HCDR1.

3.6.2.3 Pan Influenza antibodies

Selection of neutralizing antibodies recognizing subtypes from both HA groups has been a challenging goal for many years. The discovery of such a broadly cross-reactive mAb and the highly conserved HA epitope recognized by it was long considered necessary for the design of a universal Influenza A vaccine. In 2011 Corti and colleagues described the first human monoclonal antibody FI6 that

was able to neutralize members of both phylogenetic groups [75]. FI6 showed cross-reactivity to H1-H10 and H13 HAs in ELISA and neutralized multiple pseudotyped virus strains from sybtypes H1, H5 and H7 as well as various H1N1 and H3N2 Influenza A isolates. Furthermore, the antibody provided protection to mice infected with a lethal dose of A/Puerto Rico/8/1934 (H1N1) or HK-x31 (H3N2) viruses. Interestingly, based on study with FI6 mutants deficient in complement or FcR binding, the *in vivo* activity of the antibody has been identified as largely dependent on its effector functions. This explained why FI6, moderately potent in *in vitro* neutralizing assays, showed high efficacy in animal protection experiments.

Structural studies revealed that FI6v3 (an optimized FI6 variant) binds to the HA stem using both heavy and light chain. FI6 recognizes residues in the fusion peptide and the A-helix, similarly to the CR6261 and F10 antibodies. One striking difference between these antibodies is that in case of FI6 most of the binding is mediated by HCDR3. The long and flexible HCDR3 in FI6 is inserted into a hydrophobic pocket in the F subdomain of HA stem where it binds $Trp21_2$ using Phe residue. The flexibility of FI6 HCDR3 is believed to enable the binding of this Trp residue in both HA groups. In contrast, the previously described group 1 cross-reactive mAbs CR6261 and F10 use a Phe residue at the tip of their short HCDR2 loops [48, 51]. The limited flexibility of these loops most possibly does not allow the binding of $Trp21_2$ in group 2 HAs. Other differences between these mAbs involve the interaction of HCDR2 in FI6 with the carbohydrate connected with $Asn38_1$ and the contact made by LCDR1 with fusion peptide. Interestingly, the aforementioned carbohydrate, believed to decrease the binding of CR6261 and F10 to some group2 HAs, is being displaced by FI6 upon binding.

Dreyfus and colleagues have recently reported another group-1 and 2 neutralizing monoclonal antibody referred to as mAb CR9114 [79]. It has been selected from phage libraries based on human B cell genetic material. Remarkably, CR9114 showed binding to HAs belonging to group 1 and 2 of Influenza A as well as to hemagglutinins derived from both lineages of Influenza B (the Victoria and Yamagata lineage). In general CR9114 bound all tested HAs with K_D values in the low nanomolar range. Furthermore, the mAb neutralized A/New Caledonia/20/1999 (H1N1) and A/Wisconsin/67/2005 (H3N2) isolates whereas no neutralization of Influenza B viruses was shown. Surprisingly, even though not neutralizing Influenza B *in vitro*, CR9114 completely protected mice from lethal challenge with B/Florida/4/2006 and B/Malaysia/2506/2004 at concentrations 5 and 15 mg/kg. This suggests that the *in vivo* potency of this mAb is mainly dependent on its effector functions as was previously described for the other group 1 and 2 cross-reactive mAb FI6. Furthermore, CR9114 protected mice from lethal infection with A/Puerto Rico/8/1934 (H1N1) and A/Hong Kong/1/1968 (H3N2) at similar doses as reported for Influenza B experiments.

Data from crystal structure of CR9114 in complex with several Influenza A HAs shed some light on the remarkable cross-reactivity of this antibody. As expected, CR9114 interacts with HA stem. Surprisingly, the epitope is almost the same as for the group 1 cross-reactive mAb CR6261. Both antibodies use their VH 1-69 encoded heavy chains and FR3 region to recognize a hydrophobic patch on HA stem without any binding coming from the light chain. The reason for a broader CR9114 cross-reactivity comparing with CR6261 arises from several subtle differences in the mode of epitope recognition. First, both antibodies bind the $Trp21_2$ residue using a Phe on the tip of HCDR2 but in case of CR9114 a higher plasticity of this loop enables it to interact also with the $Trp21_2$ of group 2 and Influenza B HAs. Further, binding of CR9114 displaces the carbohydrate at position 38_1 in some of the group 2 hemagglutinins as well as the glycan at position 332_1 of Influenza B HA. Last, CR9114 is capable of adopting the larger Asn residue at position 49 in HA2 of group 2 viruses. This residue is occupied by Thr in group 1 HAs.

Although the epitopes of CR9114 and FI6 almost completely overlap, mAb FI6 is approaching the binding site at a different angle which probably restricts its breadth to Influenza A viruses [75]. Therefore, CR9114 is the first reported pan Influenza A and B antibody. The structural data collected in the CR9114 study are currently being applied to the design of a universal influenza vaccine.

In 2012, a study by Hu et al. reported several different group 1 and 2 cross-reactive mAbs [58]. They were isolated from human B cells and are believed to have been induced by vaccination with the 2009 pandemic H1N1 vaccine. Selected mAbs showed neutralizing activity of variable potency, depending on the tested subtype. Most of these antibodies contain VH 1-69 derived heavy chains and all of them bind the HA stem. mAbs 1F2, 1F4 and 1E1 have been further shown to recognize a linear epitope corresponding to the fusion peptide.

3.6.2.4 Molecular evolution- from germline antibodies to pan-flu monoclonals

The isolation of broadly cross-reactive, neutralizing, HA-binding antibodies has been a milestone in the field of influenza research. However, one important question remained: ‘How such antibodies are generated?’ The study by Lingwood and colleagues answered this question in detail. The molecular evolution of the VH 1-69 based, group 1 cross-reactive mAb CR6261 was investigated, starting with the germline ancestor of CR6261 (CR6261-GL) [72]. The CR6261-GL IgG molecule was not capable to bind HA. However, once expressed as surface IgM, the resulting B cell receptor was shown to be engaged by binding of HA, and tyrosin kinase signalling necessary for antibody maturation was triggered. Mutational study revealed that binding of germline VH 1-69 as an IgM molecule is dependent on the interaction of HA with Ile53 and Phe54, thus the two germ-line encoded hydro-

phobic residues at the tip of HCDR2. Furthermore, and as expected, only somatic mutations in the heavy chain were necessary to restore full activity of CR6261, confirming the lack of HA binding by the light chain. A low number of somatic mutations in the heavy chain is a characteristic feature of VH 1-69-encoded HA-specific antibodies. On average only 14 amino acids substitutions have been reported for these mAbs. The study further confirmed that only 7 of these mutations are necessary to restore full binding and neutralizing activity of CR6261. These somatic mutations are located in HCDR1 and FR3 region. The HCDR1 mutations Thr28Pro and Ser30Arg have been shown to expose the Phe29 that is buried in the structure of germline antibody. In the FR3, a Phe is introduced at position 74, and together with Phe29, it makes essential contacts with a hydrophobic patch on HA stem.

A similar evolutionary study has been conducted for the group 1 and 2 cross-reactive FI6 antibody that is encoded by V_H 3-30 and V_L 4-1 germline genes [75]. FI6 was compared with its germline-reverted ancestor FI6-GL, closely related but only group 1-specific FI370, and FI6/370-BP, a hybrid antibody that contains all the somatic mutations shared between FI6 and FI370. FI6, FI370, FI6/370-BP and FI6-GL bound HA from group 1 whereas only FI6 could strongly bind a hemagglutinin from phylogenetic group 2.

Based on these data it can be speculated that the VH 1-69 and the combination of VH 3-30 and VL4-1 germline genes evolved as universal pattern recognition molecules binding to conserved epitopes on influenza hemagglutinin [72, 75]. It seems that the evolution of 2 of such germlines was necessary as some individuals contain a homozygous mutation Phe54Leu abolishing the binding of VH 1-69 to HA.

3.6.2.5 Heterosubtypic antibodies recognizing epitopes outside HA stalk

At least one broadly cross-reactive, neutralizing antibody binding an epitope outside HA stem has been reported to date. mAb S139/1 was shown to react with H1, H2, H3, H5, H9 and H13 subtyped viruses whereas neutralization could be demonstrated for H1, H2, H3 and H13-expressing viruses [80]. No neutralization was found for H5, H9 and Influenza B isolates. S139/1 prevented hemagglutination of erythrocytes suggesting that the antibody neutralizes the virus by blocking HA receptor-binding site. Escape variants generated by extensive passaging of H1N1, H2N2 and H3N2 viruses in the presence of S139/1 indeed had mutations at positions 156, 158 and 193 (H3 numbering). This maps the recognized epitope at the tip of globular head region in close proximity of receptor binding site. Thus, the S139/1 epitope resembles more a unique, conserved antigenic site. Indeed a partial overlap between the site bound by S139/1 and antigenic site Sb (H1) or B (H3) has been reported.

3.7 Influenza A treatment and prevention- past, present and future

Each stage of the Influenza A virus 'life' cycle has been studied in terms of inhibition of viral replication using various compounds (Figure 11). In this chapter a brief overview of the currently marketed and experimental influenza drugs is given. Further, currently available vaccines used for influenza prevention and recent advances in this field are reported.

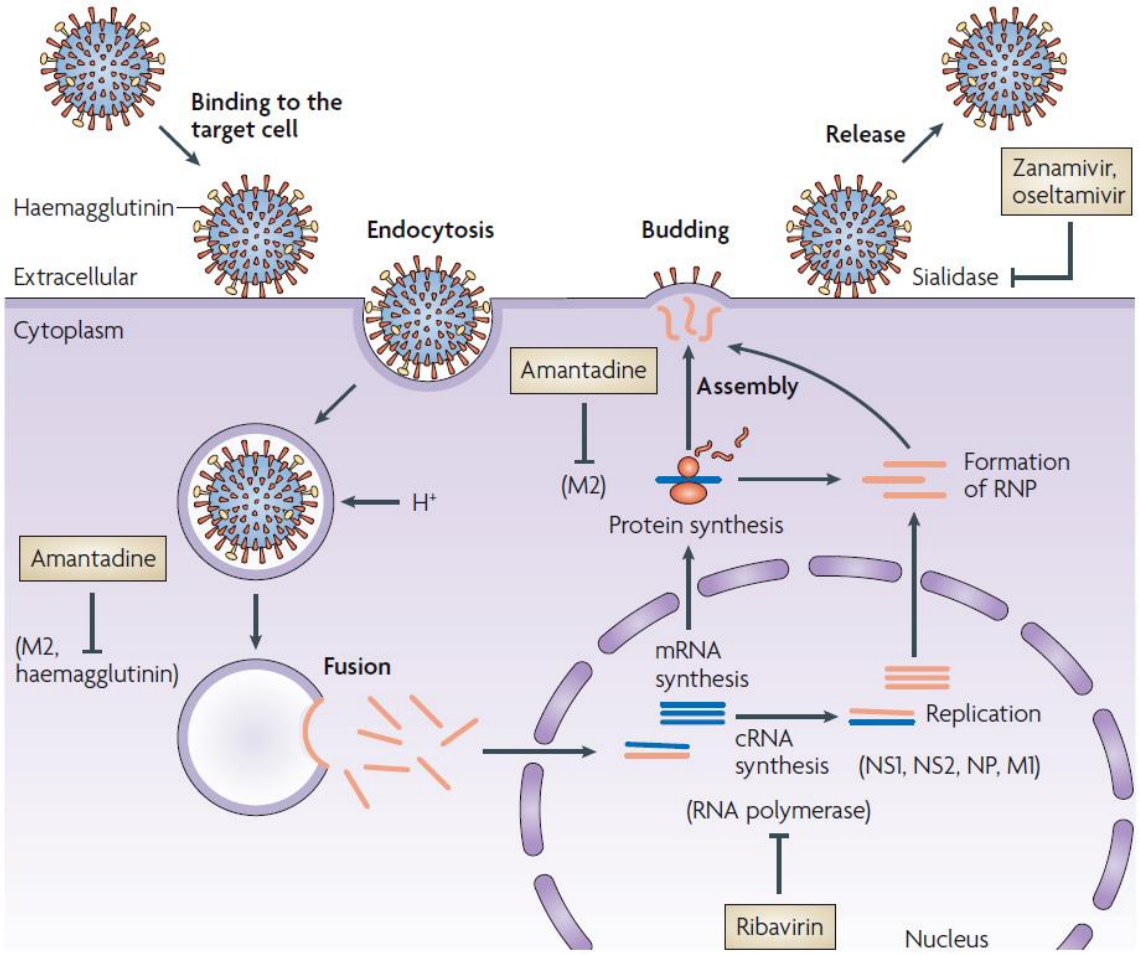


Figure 11. Inhibition of Influenza A virus at different stages of its 'life' cycle. The interaction of Influenza A virus with host cell receptors is mediated by hemagglutinin. Once bound the virus is internalized into endosome. The low pH of endosome triggers the membrane fusion process and virus disassembly. vRNPs are further transported to the nucleus where the viral replication and mRNA synthesis occurs. The mRNA is next utilized to express viral proteins that interact with each other and vRNPs to form new virions. Each step of the infection and replication cycle can be targeted by drugs. Examples of such drugs are depicted in grey boxes. From [81].

3.7.1 Small molecule inhibitors

3.7.1.1 Neuraminidase inhibitors

Oseltamivir and zanamivir, two neuraminidase inhibitors, are the only marketed influenza drugs besides the M2 inhibitors. Oseltamivir and zanamivir have been developed based on the structure of 2-deoxy-2,3-dihydro-*N*-acetylneuraminic acid- the prototype NA inhibitor that was lacking *in vivo* activity [1, 82]. Both drugs interact with the NA receptor binding pocket by structurally mimicking sialic acid and show activity against Influenza A and B viruses. The effectiveness of these drugs largely depends on the time of administration. Studies showed that the treatment has to start within 48 hours from the onset of symptoms in order to benefit from the therapy [83]. Oseltamivir is orally administered whereas zanamivir is inhaled. Since both drugs are interacting with Glu276 in NA, mutations in close proximity to this residue confer resistance. Several different resistant variants have been reported to date. The H274Y and R292K mutants are insensitive to oseltamivir with the latter one moderately affecting the binding to Zanamivir [84]. Residues H274 and R292 interact with the aforementioned Glu 276 enabling its rotation necessary to accommodate the large hydrophobic side chain in oseltamivir [83]. The occurrence of oseltamivir-insensitive virus variants changes during each season, and is variable among different subtypes [47]. In 2008-2009, almost 100% of seasonal H1N1 isolates were resistant whereas the H3N2 and Influenza B viruses remained sensitive [85]. In contrast since 2010 the oseltamivir resistance in all circulating human viruses has been low (including H1N1 isolates). Resistance to zanamivir has been marginal till now and oseltamivir-resistant isolates have been sensitive to zanamivir.

Recently two other neuraminidase inhibitors have been approved for use in humans in treatment and prevention of Influenza A and B infections [86]. Peramivir and laninamivir are modified versions of oseltamivir and zanamivir [83]. Peramivir binds NA in a similar way to oseltamivir and therefore H274Y-bearing virus mutants are also resistant to peramivir. However, the resistance to peramivir is less pronounced than to oseltamivir. For laninamivir, no naturally occurring resistant variants have been reported so far. Nevertheless, it is believed that this compound, structurally similar to zanamivir, will not be active against zanamivir resistant viruses.

3.7.1.2 M2 inhibitors

M2 protein consists of 4 transmembrane helical regions and a C-terminal cytoplasmic helix [84]. The transmembrane helices form a pore that transfers protons inside the virus particle at low endosomal pH. This acidification of the virus lumen leads to virus uncoating and RNP release. Proton

transfer is mediated in a pH-dependent, coordinated action of His37 and Trp41 residues. Amantadine and rimantadine (adamantine derivatives), two M2 ion channel inhibitors, have been approved for treating humans and are in use since many years. X-ray structure and NMR studies showed a different mode of action for these compounds. Amantadine binds inside the proton channel, close to Ser31 residue, physically blocking the pore. Rimantadine interacts with outer surface of the M2 helices, in the close proximity of Trp 41. It is believed to lock M2 in the 'closed', inactive conformation.

Several different mutations in M2 (L26F, V27A, A30T, and S31N) are responsible for viral resistance to amantadine and rimantadine [87]. These mutations have propagated in circulating Influenza A viruses during last 2 decades. As a result, 100% of human isolates tested in 2009 showed resistance to both drugs. Emerging resistance and CNS side effects related to use of these drugs rendered amantadine and rimantadine inadequate for treatment of Influenza A infections in humans. However, several new adamantine derivatives and non-related compounds blocking the action of M2 have been identified in recent years. Among the adamantine derivatives, one of the most potent is Spiro[piperidine-2,2′-adamantane]. This compound and its modifications have been shown to block the M2 in a similar manner as amantadine, and were also active against some amantadine resistant mutants. In the group of non-adamantine compounds the 2-[3-azaspiro(5,5)undecanol]-2-imidazoline (BL-1743) and its derivatives showed significantly higher potency than amantadine but were not able to neutralize adamantine resistant variants. Nevertheless, BL-1743 is being used now as starting point in the design of drugs that can overcome reported M2 resistance, especially to the predominant S31N mutation.

3.7.1.3 Hemagglutinin inhibitors

Tert-butylhydroquinone (TBHQ), is a small aromatic compound that is effective in neutralizing Influenza A viruses [88]. However, action of TBHQ is limited to group 2 viruses. TBHQ interacts with hemagglutinin and blocks the structural rearrangement of the molecule that is necessary for membrane fusion. X-ray crystallography revealed that TBHQ binds to the HA stem between CD-helices of two neighboring HA monomers, and slightly above epitopes recognized by CR6261, F10, FI6 and CR9114 antibodies. Detailed analysis of TBHQ epitope showed structural differences at this site between group 1 and 2 HAs. The lack of binding to group 1 viruses was attributed to an extra turn made by A-helix in close proximity to TBHQ binding site. Such turn is only present in group 1 HAs, explaining the limited cross-reactivity of this compound.

3.7.2 Monoclonal antibodies

A considerable number of broadly cross-reactive antibodies binding influenza hemagglutinin has been isolated during the recent years [89]. The aforementioned mAbs CR6261, F10, FI6, CR8020, CR9114 etc. proved to be effective in protecting animals lethally infected with corresponding viruses. This has raised the hope of using such antibodies in a therapeutic or preventive setup in humans, too [90]. As more and more Influenza A isolates display resistance to currently marketed small-molecule inhibitors, these antibodies may be the only alternative in treatment of influenza infections. Moreover, such monoclonal antibodies have several advantages over chemical compounds used as drugs at the moment. First, they target highly conserved epitopes. For some of these mAbs, generation of escape mutants was not possible whereas for others it required extensive passaging of virus in presence of limiting dilution of given antibody [48, 51, 75, 79]. It is conceivable that using a cocktail of multiple mAbs, e.g CR9114 with FI6, would be effective against any emerging virus and no mutations could confer resistance to such treatment. Second, the bioavailabilty, pharmacokinetics and side effects for these biologicals may be improved compared to oseltamivir or zanamivir. Since these antibodies were isolated from human combinatorial antibody libraries or human B cells, they should be well tolerated. Last, the number of HA cross-reactive mAbs reported to date already vastly exceeds the number of currently available drugs giving the possibility to adjust the treatment to patient's needs. On the other hand the manufacturing process of monoclonal antibodies is more tedious and expensive compared to that of small molecules. However, it has to be noted that recent advancements in mAb production (improved expression yields, decreased cost) provide hope for introducing such antibodies on the market.

3.7.3 Computationally designed proteins

In 2011, Fleishman and colleagues described a completely new approach for generating proteins binding to the conserved epitopes on influenza A HA [91]. The group used computational design to identify residues and scaffolds complementary to chosen HA epitope. The new binding proteins have been constructed in a two-stage process. First, a set of disembodied amino acids have been docked into the epitope in energetically favorable configurations. These amino acids have been chosen to provide interactions common in protein-protein complexes. Second, sets of selected disembodied amino acids have been applied to complementary scaffolds. In total 88 different designs have been tested resulting in 2 HA binding proteins, HB36 and HB80. Both proteins have then been subjected to affinity maturation that increased their K_D values to the low nanomolar range. Crystal structure showed that HB36.3 (a matured variant of HB36) interacts with the HA stem and the recognized epitope closely resembles the computationally designed one. Further, this epitope is overlapping the

one recognized by CR6261. In a follow up study from 2012, both proteins have been further improved in terms of affinity and cross-reactivity however no interaction with group 2 HAs was reported [92]. Noteworthy, the F-HB80.4 variant neutralized virus particles of recent H1N1 subtypes. These data bring hope into fast development of proteins with desired properties. Such proteins, inexpensive in production, could be potentially used to treat broad spectrum of Influenza A infections.

3.7.4 Influenza A vaccines

3.7.4.1 Seasonal Influenza vaccines

First prototypes of seasonal influenza vaccines have already been described in 1930s [1]. At this time, culturing of Influenza A strains in embryonated hen eggs has been established, and provided large quantities of viral material necessary for vaccination. During World War II, first experimental inactivated vaccines have been administered to US military personnel starting the era of widespread vaccination against flu.

Nowadays, inactivated seasonal vaccines are based on 3 types of viruses: an H1N1, an H3N2, and a B virus. Influenza strains used in these trivalent vaccine are annually selected, based on the analysis of circulating viruses and a prediction of the viruses to be predominantly circulating in the next influenza season [93]. It is crucial to properly match the virus as this type of vaccines only provide protection against the inoculated or very closely related strains.

For the production, selected viruses are grown in embryonated hen eggs. The allantoic fluid containing virus particles is then harvested, and particles are purified using either centrifugation or column chromatography [94]. The virus is next inactivated with formalin or beta-propiolactone and treated with detergent to solubilize the membrane. Such trivalent inactivated or split vaccine (TIV or SV) is currently used as standard for vaccination against seasonal influenza.

Recent advances in the production of inactivated vaccines

There have been several improvements in the process of vaccine production over the years. First, production yields have been increased by growing reassortant viruses containing the HA and NA antigens from selected seasonal isolate in the genetic background of the robust H1N1 A/Puerto Rico/8/1934 strain [1, 94, 95]. Second, the introduction of additional virus purification techniques during the manufacturing process has provided vaccines that contain less by-products and thus have a lower incidence and severity of side effects. Third, new production techniques such as removing

the polybasic region enabled to amplify strains that normally would be lethal to chicken embryos [1]. These techniques make vaccine production, e.g. against highly pathogenic, potentially pandemic H5N1 viruses possible. Alternatively, amplification of highly pathogenic viruses can now be performed in cell culture. In 2012 FDA approved the use of Flucelvax, the first influenza vaccine prepared by growing viruses in cell culture [96]. This approach has several advantages over the egg-based manufacturing process: it is faster, it yields viruses that do not drift genetically from the parental strain used as inoculum and it is not dependent on seasonal availability of eggs and SPF flocks that lay these eggs. One of the last improvements in the current process of seasonal vaccine preparation has been made in the field of split vaccines. In 2013 FDA approved Flublok, the first vaccine composed of recombinantly expressed hemagglutinins from each H1N1, H3N2 and B strains [97]. Interestingly, the vaccine is effective against all tested, circulating human isolates and not only the vaccine strains.

Efficacy of inactivated vaccines

Efficacy of seasonal, inactivated vaccines ranges from 45 to 85% [1, 97, 98]. This value depends on the type of vaccine and several other factors, for instance the degree of the immunological match between the vaccine strain and the circulating viruses. Further, better immune responses are observed when the vaccinated individual has previously been infected with a virus of the same subtype. In case of the introduction of a new subtype into the human host, vaccination against this subtype has to be performed at a higher dose [99]. In general, protection from virus challenge has been correlated with antibody titers to HA. Although responses to NA and activation of CTLs can also be detected, these two factors contribute much less to protection [1]. Furthermore, the breadth of HA-specific antibodies induced by vaccination is influenced by the disease history. If the individual is seronegative, the induced HA antibodies are mostly strain-specific. In contrast, vaccination of subjects that have previously experienced multiple influenza infections is more likely to elicit broadly cross-reactive sera. Moreover, antibody breadth may depend on the vaccine formulation as it was mentioned above for Flublok. HA antibody titers decrease significantly within the first year after vaccination. This observation, combined with the limited protection provided against antigenically drifted seasonal strains, requires that vaccination should be performed annually.

Live attenuated vaccines

Live attenuated vaccine (LAV) has been first introduced into US market in 2003 under the brand name 'Flumist' [100]. In 2011, FluMist was also approved in the European Union under the

market name 'Fluenz' [101]. These vaccines have been developed to overcome some of the limitations of inactivated vaccines. One major drawback of inactivated vaccines is that they have a lower efficacy in those groups that are at high risk of influenza infection (e.g. the elderly)[1]. Another disadvantage is that inactivated vaccines induce a very limited antibody repertoire, providing protection only to the vaccine or closely related strains. Live attenuated vaccines have been shown to overcome some of those problems. This property can be partially attributed to the fact that live vaccines, besides stimulating antibody response, also induce T cells [102]. Furthermore, the intra nasal application of LAV potently stimulates the immune response at the primary site of virus entry and replication [103]. This seems to provide a better protection than the systemic immune response induced by inactivated vaccines.

LAV consists of viruses whose growth has been attenuated by multiple passages at a lower temperature (25^{o}C). An example of such virus is A/Ann Arbor/60/60 (H2N2) strain [1]. This virus can not efficiently replicate at the usual body temperature of 37-38 ^{o}C that is normally permissive for wild type viruses. To facilitate the production of the vaccine, a universal manufacturing system based on A/Ann Arbor/60/60 (H2N2) has been designed. In this system, the aforementioned H2N2 virus is reassorted with selected seasonal strains. The resulting vaccine strain contains the genetic background of A/Ann Arbor/60/60 with NA and HA genes from the circulating strain.

Multiple clinical studies proved the superior performance of LAV over inactivated vaccines especially in children. The work by Belshe and colleagues showed a high efficacy (93%) of LAV in seronegative children 15 to 71 months of age [104]. This knowledge has further been extended in a clinical study from 2008. Here a comparison with inactivated vaccine showed that LAV vaccinated patients aged 2-5 years had 52.5% and 54.4% less cases of influenza illness following infection with a matched or mismatched virus, respectively [105]. Moreover, a study from 2010 proved that the vaccine provides a certain level of cross-protection against drifted and mismatched Influenza B strains in children 6 months to 6 years [106]. Currently, several other live attenuated vaccines against influenza are being developed with an H5N1 vaccine being of major interest [107, 108].

3.7.4.2 Experimental vaccines

Although widely used since decades, and substantially improved in recent years, traditional seasonal influenza vaccines are inefficient. Their major limitation is that they do not provide heterotypic protection. This implies that they have to be formulated annually based on predictions of the upcoming seasonal strains [93, 94]. As a result, vaccine delivery is significantly delayed and the vaccine itself may not be effective if the vaccine strain is a poor match with the actually circulating

strain. Many studies have been performed to address this issue and to produce a universal flu vaccine. Induction of antibodies as well as T-cell response has been tested [109]. Furthermore, various influenza proteins have been included in experimental formulations. A brief overview of these is provided below.

3.7.4.2.1 M2e vaccines

M2e, the external domain of M2 protein, has long been considered as a candidate for the development of a universal Influenza A vaccine. This short stretch of 18-24 amino acids is present on virus surface and is highly conserved amongst all Influenza A viruses [110, 111]. Thus, inducing a robust immune response to it could possibly provide a heterosubtypic protection. Although there are some differences in amino acid composition between the human and avian-type of M2e, it has been shown that serum specific for the first type of M2e is also cross-reactive to the second type.

Even though theoretically promising, M2e used as an antigen has one major limitation: the lack of immunogenicity [110]. This issue has been successfully addressed by incorporating multiple M2e molecules into virus-like particles (VLPs) in connection with highly immunogenic proteins. A good example here is the fusion of M2e with Hepatitis B core (HBc) protein [112]. VLPs based on this construct contain approximately 240 HBr-M2e fusion proteins organized in 120 dimeric spikes. Such VLPs proved to induce a strong immune response in mice as evaluated based on high serum M2e-specific antibody titers. Moreover, mice vaccinated with such immunogen were protected against virus challenge even after intranasal (i.n.) vaccine administration. The mechanism of M2e-based protection has long been unclear. It was known that antibodies induced against M2e are not neutralizing. M2 protein containing M2e is incorporated into cell membrane during virus replication cycle. Due to high density of surrounding glycoproteins, surface exposed M2e is possibly not accessible to B and T-cells. However, it has been proven in several studies that surface exposed M2e is bound by immunogen-induced antibodies [110]. These antibodies activate antibody-dependent cell cytotoxicity (ADCC) leading to the elimination of infected cells and thus virus clearance. Phase I clinical trial performed by Acambis Inc (currently Sanofi Pasteur) using HBr-M2e VLPs (ACAM-FLU-A vaccine) showed that high titers of M2e antibodies are induced after 2 immunizations in almost all subjects. Potency of this and other M2e based vaccines in humans is currently studied.

Several different approaches to M2e-based vaccines have been employed. In the study by Price et al. entire M2 and NP genes have been incorporated into an adenoviral vector [113]. These recombinant adenoviruses have been used as i.n. vaccine in mice and provided protection against viruses of the H1N1, H3N2 and H5N1 subtypes. Detailed analysis showed induction of high antibody

titers and specific T-cells as possible reasons for the reported clearance of Influenza A infections. In another investigation, Powell and colleagues used nanoparticles that mimic natural pathogens and induce potent immune response in absence of adjuvants [114]. These nanoparticles were coated with several different layers of oppositely charged polypeptides with M2e peptide included in the external layer. Mice immunization with M2e nanoparticles elicited high titers of M2e specific antibodies. Furthermore, the same study using several different nanoparticle constructs with various immunogens showed that there is no antibody response to the particles themselves. This is a significant advantage comparing to other delivery vectors like the aforementioned adenoviruses, as it enables multiple vaccinations without antibody-related clearance of the vector [115, 116]. Furthermore, nanoparticles were shown to be internalized by dendritic cells (DC) and to potently induce CD4 and CD8 T-cells specific to the delivered antigen [114]. Thus, this nanoparticle-based approach seems to be very promising in designing a universal Influenza A vaccine but requires further investigation.

3.7.4.2.2 Vaccines based on pseudoparticles

Another approach to the design of a universal Influenza A vaccine has been reported by Powell and colleagues in 2012 [102]. They used pseudotyped virus based on the H1N1 A/Puerto Rico/8/1934 strain as immunogen. These pseudoparticles (termed S-FLU) contained the complete genome of the virus but were not capable to undergo a complete replication cycle. Attenuation of infectivity was achieved by introduction of multiple mutations into the HA gene that rendered it dysfunctional. Therefore, pseudoparticles were amplified in 293T cells transfected in parallel with a plasmid containing an intact HA sequence to complement *in trans* and to enable budding of infective virus. Although expressed in cytoplasm, the defective HA was rapidly degraded and available for surface display to T cells. Mice vaccinated twice with S-FLU were completely protected from challenge with H1N1 and H3N2 viruses. The protection was later attributed to activation of antigen-specific T cells. Furthermore, no significant antibody response to HA was detected unless large doses of S-FLU were used. It has also been proven that the mutated HA gene is not crucial for eliciting protective immune response. The modified version of S-FLU, containing eGFP instead of mutated HA gene, performed comparably well to the HA construct.

The S-FLU presents multiple advantages over other vaccine designs. First, it elicits heterosubtypic protection. Second, S-FLU does not present the risk of providing a novel HA gene that could be reassorted with circulating seasonal strains. This has been a particular concern in case of life attenuated vaccines. Third, even though S-FLU is using a similar approach as adenovirus-based gene delivery, much lower doses of S-FLU pseudoparticles are necessary for immunization. In the study, doses

4 orders of magnitute lower than for similar adenovirus therapies have been sufficient to elicit strong immune response. This further emphasizes the potency of such immunogen.

3.7.4.2.3 Vaccines based on recombinant hemagglutinin

The discovery of broadly cross-reactive antibodies binding conserved epitopes in HA stem has become a foundation for the design of a universal influenza vaccine. In recent years several different approaches to this subject have been reported [93, 117]. One common feature for all of them is that they focus on exposing the previously mapped conserved stem epitopes. Isagawa's and Palese's groups, have extensively studied multiple HA based constructs in terms of eliciting heterotypic immune response [117-121]. In a study from 2010, Palese's group reported vaccination with a set of 'headless' hemagglutinins that have been constructed by removing the HA1 portion present between Cys 52 and 277 residues [117]. These construct contained the stem related HA1 and all HA2 amino acids connected with various linkers. The fold of these proteins has been confirmed using stem-reactive antibodies and the most promising candidates have been used for mice immunization. The vaccination schedule consisted of intra muscle electroporation of plasmid DNA containing sequence of a particular construct followed by injection of VLP containing the related protein. H1-based headless HA vaccine induced antibodies cross-reactive to H1, H2 and H5 whereas H3-based headless HA elicited sera only reactive to the vaccine strain. Although induced sera had limited cross-reactivity and did not neutralize the virus *in vitro*, protection of mice challenged with H1N1 (A/Puerto Rico/8/1934) has been reported for the H1 construct. These data suggested that this type of design is insufficient. Therefore a new approach has been tested. In a study from 2013 several constructs, all containing the same stalk (from H1 PR8) but different heads (H9, H6, H5), were tested (chimeras cH9/1, cH6/1, cH5/1) [119]. The idea behind this setup is to avoid inducing antibodies towards the head portion that changes at each vaccination and focus the antibody response on stalk that stays invariant at each step. First, mice were electroporated with DNA containing the cH9/1 chimera. Later, animals were boosted with subsequent injections of cH6/1 and cH5/1 as soluble proteins. The vaccinated mice were fully protected against challenge with H1N1, H5N1 and H6N1 but no protection was reported for H3N2. Furthermore, binding to H2 HA and neutralization of H2N2 virus with IgG from vaccinated mice was shown. As H2 subtype was not used in vaccination it was speculated that the elicited antibodies recognize the conserved HA stem.

This and several other studies have shown that using only the stem of HA as an immunogen might be not sufficient to elicit cross-reactive sera and to provide a broad protection against viral challenge. This might be related to some subtle structural changes that may occur in stem once it is expressed without the head. These changes, although not detectable in antibody-based assays, may

somehow decrease the breadth of vaccine elicited sera. Therefore other modifications of the system have been applied. In the study by Wang and colleagues only a short stem-derived peptide has been used as immunogen [120]. This peptide consisting of HA2 amino acids 76-130 (termed LAH for the presence of long α-helix) of H3 has previously been described to be recognized by cross-reactive mAb 12D1. To enhance the antigenicity and extend serum half-life of the peptide, LAH was conjugated to keyhole limpet hemocyanin (KLH) and acetylated. Mice vaccinated with LAH-KLH construct had sera cross-reactive to H1, H2, H3, H5 and H7 subtypes in ELISA. Further, they were completely protected against H3 and partially protected against H5 challenge. Although no protection was seen against H1 virus the disease was delayed in the vaccinated group.

3.8 Phage display- a tool to select antibodies with desired properties

Phage display was first described by Smith et al. in 1985, and is a widely used and powerful technology to select high affinity molecules with desired properties from large repertoires [122]. Initially only small polypeptides could be displayed on the surface of filamentous phage particles [123]. This changed during the last two decades. Many new, optimized scaffolds of displayed proteins were designed that enabled recognition and binding of almost every target antigen (e.g. proteins, nucleic acids) [124-126]. For phage display, a filamentous phage (e.g. M13) is modified to display a binding molecule (e.g. Fab antibody fragments, polypeptides) on its surface for which it also carries the genetic information [127]. Such molecule is fused to one of the phage coat proteins in a way that it can make interactions with the target of interest. During selection process, phage clones presenting binding moiety for the desired target molecule are enriched. The sheer size of phage display libraries enables screening of up to 10^{10} different variants of binding molecules in few relatively simple steps. Up till now phage display has been successfully applied in many fields, e.g. selection of high affinity synthetic antibodies, selection of molecules with improved stability and folding, selection for enzymatic activity.

During the phage display selection process, the target molecule is usually immobilized on solid surface like magnetic beads or plates, and incubated with a phage library that displays a large variety of potentially binding molecules [122]. Due to the design of a phage vector only one binding specificity is displayed per phage particle. After incubation phages that do not bind the antigen are washed away. The remaining bound phages are then eluted, amplified in *E. coli* and used for the next selection round. Usually 3 rounds of selection are sufficient to obtain clones binding tightly to target molecule. Each round may differ in the target molecule concentration, time of incubation or wash stringency. By controlling these parameters antibodies with desired properties can be obtained. The selection results in a set of phage clones that are reamplified separately in bacterial host and further

tested for binding to the relevant antigen e.g. in ELISA. Due to the fact that each phage clone carries the genetic information encoding the displayed e.g. Fab fragment positive clones can be further used to amplify the sequence of binding molecule, clone it to another vector and express as soluble protein/peptide. These can be later evaluated in various experiments like neutralization assays. If the obtained molecule only weakly binds the relevant target further affinity maturation using different techniques (e.g. error prone PCR, site directed mutagenesis) can be applied. Moreover, molecules interacting with an exact area on the antigen may be obtained by introducing appropriate antigen design (e.g. sterically blocking access to irrelevant surface) [128].

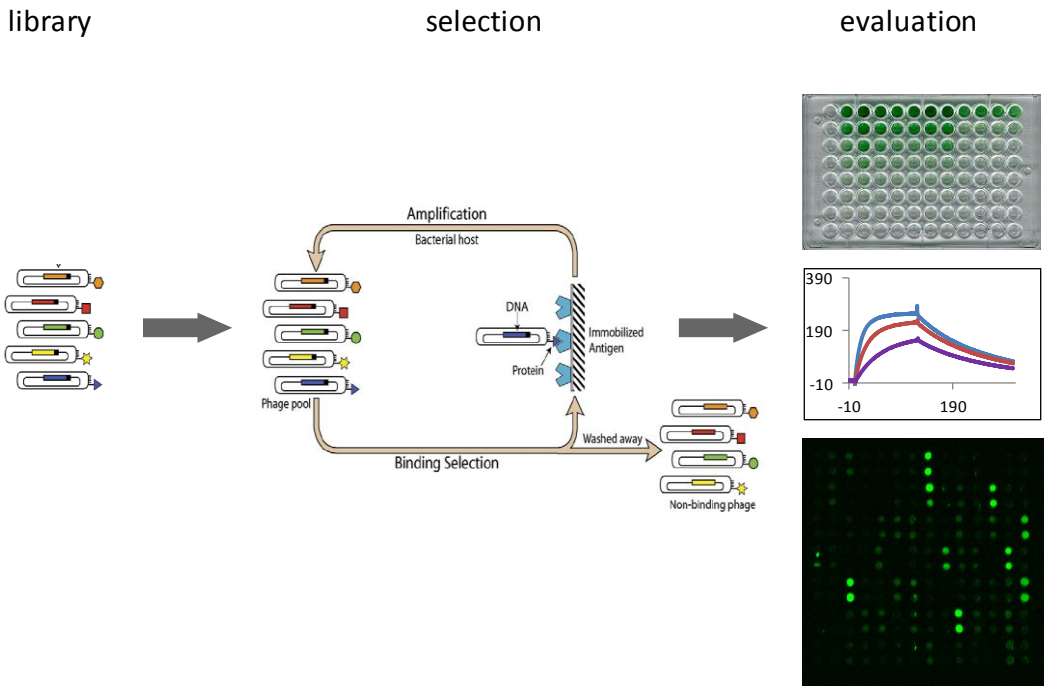


Figure 12. Example of phage display selection process. In the phage selection a library of phages displaying binding molecules (e.g. Fabs) is combined with the antigen of interest immobilized on solid surface. Phages in the library display molecules with different binding specificities (e.g. various CDR composition in Fab fragments). Phage clones that interact with the immobilized target via the Fab fragment are captured, eluted, reamplified in bacterial host and used in the following selection round. The resulting clones can be further evaluated for their binding properties (as phage particle or as purified Fab) using different assays (e.g. ELISA, SPR, microarray). Modified from [127].

4. Results

4.1 Alternative recognition of the conserved stem-epitope of influenza A hemagglutinin by a V_H3-30-encoded heterosubtypic antibody

This is a manuscript in preparation. I contributed in the following way: I prepared the phage display library by cloning the genetic material from isolated B cells; mutated, cloned, expressed, purified and biotinylated the H2 hemagglutinin used for panning; performed selection; expressed and purified some of the hemagglutinins used in subsequent assays; validated mAb 3.1 in binding and neutralization assays; expressed mAb 3.1 for crystallography study; performed in vivo protection assays. I also participated in figure preparation.

Alternative recognition of the conserved stem-epitope of influenza A hemagglutinin by a VH3-30-encoded heterosubtypic antibody

Wyrzucki A[1], Dreyfus C[2], Kohler I[1], Steck M[1], Wilson IA[2], Hangartner L[1]

[1]Institute of Medical Virology, University of Zurich, Switzerland; [2]The Scripps Research Institute, La Jolla, USA

Summary

Using immobilized hemagglutinin from A/Japan/305/1957(H2N2) in phage display, V_H3-30-encoded human monoclonal heterosubtypic antibody mAb3.1 was isolated. It was found to neutralize influenza viruses from the H1a clade but had no activity against viruses of the H1b clade or of phylogenetic group 2. The crystal structure of mAb 3.1 revealed that it contacts the same hydrophobic groove in the stem of the HA protein as most other heterosubtypic antibodies, and that primarily engages residues of its heavy chain for binding. However, in contrast to the closely related mAb FI6 that almost exclusively relied on residues of CDRH3 for binding, mAb 3.1 was found to involve residues from CDRH1, CDRH3 and FR3. Thereby, CDRH1 of mAb 3.1 adopts an α-helical structure conformation and engages in very similar hydrophobic interactions with HA as the de novo *in silicio* designed and affinity matured artificial protein HB36.3.

Introduction

Hemagglutinin (HA), the surface protein responsible for receptor attachment and entry of influenza A viruses, exists in 17 distinct subtypes [1] that can be divided into two separate phylogenetic groups. HA assembles as a trimer and is initially synthesized as an inactive form (HA0) that is processed to its active form by cleavage into covalently linked HA1 and HA2 subunits. In the viral spike, the apical globular head that contains the receptor-binding site is made of the HA1 subunits while the stalk region containing the fusion machinery is built by HA2 subunits combined with the N and C-terminal ends of HA1. Antibodies elicited during infection and immunization bind to highly antigenic domains surrounding the apical receptor binding site on HA1, and typically interfere with receptor binding [2-5]. As these antigenic sites are also subject to the highest antigenic variation, most antibodies recognizing influenza HA are highly strain-specific, and only recognize the eliciting or closely related virus strains.

Currently, only influenza viruses of the H1 and H3 subtypes circulate in the human population. Yet, cases of zoonotic infections with avian or swine viruses are reported on a regular basis, indicating that, to some degree, the species barrier is permeable for influenza viruses [6-9]. Although most of these infections are relatively benign, zoonotic infections with highly pathogenic avian influenza A viruses (HPAI) of the H5N1 subtype are considered to end lethal for more than 60% of all infected individuals. Although this number is likely to be an overestimate due to non-reporting of sub-clinical and mild cases, it nonetheless underlines the urgent necessity to be prepared for potentially lethal zoonotic infections. Moreover, as zoonotic infections are the origin of novel pandemic strains, prophylactic or post-exposure treatment of potentially exposed individuals could prove helpful in restricting the early spread of emerging viruses. Recent emergences of zoonotic H7N9-bearing viruses that are probably capable of human-to-human transmission [10] further stress the need for such first line defense treatments. As the usefulness of the currently available drugs is rapidly decreasing, alternative treatments e.g. with passively transferred heterosubtypic antibodies, i.e. antibodies that neutralize more than one subtype (or even genus) of influenza, could offer an efficient alternative to small molecule inhibitors. Moreover, the ability to induce heterosubtypic antibodies by immunization would allow for the development of a pan-flu vaccine and would make the annual reformulation and application of the seasonal vaccine obsolete.

The first heterosubtypic monoclonal antibody (hmAb) isolated, C179, was generated in mice that were hyperimmunized with an H2N2-expressing human virus around 20 years ago [11, 12]. Meanwhile, several human heterosubtypic antibodies have been isolated and their epitopes characterized [13-20]. With the exception of the exclusively group 2-specific heterosubtypic antibody CR8020 that binds to a more membrane proximal epitope, all other heterosubtypic antibodies recognize approximately the same epitope on the stem of influenza A hemagglutinin (HA). This epitope, a hydrophobic groove that is framed by residues 18-52 and 290-330 of HA1 in combination with 1-21 and 38-60 of HA2 is very conserved amongst all subtypes of influenza A, and, to some degree, also shared with viruses of the B genus [19].

Quite interestingly, several antibodies binding to this epitope are encoded by the V_H1-69 (F10, CR6261, CR9114, 3C4) or the V_H3-30 (FI6, 1C4) germline genes, and mostly only bind to HA subtypes of phylogenetic group 1 (H1, H2, H6, H8, H9, H11, H12, H13, H16). Only recently, monoclonal heterosubtypic antibodies capable of neutralizing viruses from both phylogenetic groups have been described [16, 19, 20]. To date, six human heterosubtypic antibodies that are not encoded by the V_H1-69 or V_H3-30 germline genes have been published (PN-SIA49, 1E1, 1F2, 1F4, 1G1, 3E1)[17, 20]. Five of these antibodies use the V_H3-23, one the V_H4-4 heavy chain germline gene [17, 20]. Also these antibodies are either specific for phylogenetic group 1 [17], or can neutralize viruses from both phyloge-

netic groups [20]. So far only one antibody that exclusively recognizes HA subtypes from phylogenetic group 2 has been described [15].

V_H1-69-encoded heterosubtypic antibodies are frequently isolated, and primarily use their heavy chain to contact the HA protein. Although germline encoded V_H1-69 antibodies devoid of somatic hypermutation do not recognize soluble HA, they can trigger B cell receptor signaling when engaged by HA as IgM molecule on the surface of B cells [21]. From the available crystal structures, it can be deduced that positions 49, 111 and 21 in HA2, as well as position 18 and the presence or absence of a glycan at position 38 of HA1 account responsible for the main differences between the two phylogenetic subgroups [13, 14, 16, 19]. Latter has been shown to be flexible enough to accommodate binding of an antibody in its proximity [16, 19]. Antibodies capable of overcoming these differences have been demonstrated to achieve this by the flexibility of their complementarity-determining region (CDR)[16, 19]. V_H3-30-encoded FI6, for instance, is characterized by a particularly long HCDR3 loop (22 aa) that provides the hydrophobic residues required for the crucial contacts with the hydrophobic groove in the stem of HA (i.e. Leu100A, Tyr100C, Phe100D Trp100F). In contrast, murine mAb C179 and all V_H1-69-encoded antibodies, including CR9114, contact this groove with hydrophobic residues from all HCDRs of their heavy chain [14, 19]. Additional contacts can assist the interaction of the heavy chain with the hydrophobic groove, and can either include 3 residues of LCDR1 (Phe27D, Asn28, Tyr29; FI6), or from FR3 (D72, I or D73, F74; CR6261 and CR9114).

Results

Isolation and characterization of monoclonal antibody 3.1

Using RNA isolated from mature B cells of a healthy donor, a Fab phage display library was prepared and used for panning against trimeric baculovirus-expressed HA from A/Japan/305/1957(H2N2) [3] that was reversibly immobilized on magnetic beads. The H2 subtype was chosen for two reasons: First, antibody mapping suggested that there is an antigenic site in the stem of this subtype protein that is not present in other human HA subtypes [2, 3]. Second, the donor of the B cells used for the preparation of the phage library was born in 1976, and should therefore be immunologically naïve to the H2 subtype that ceased circulating in humans in 1967. Consequently, antibodies isolated from this donor that are capable of binding H2 are *bona fide* heterosubtypic antibodies.

After four rounds of panning, 13 clones were selected for further characterization. All clones possessed virtually the same heavy chain paired to different light chains. The HCDR3 amino acid (aa)

sequences (N-CARDLGGYFIRGIMDVW-C) found in all heavy chains suggests a common ancestor arising from a single IGHV3-30*04, IGHD3-9*01 to IGHJ4*01 recombination event. This notion is further supported by the observation that 9 out of 13 heavy chain sequences are completely identical, with the remaining clones only displaying a maximum of four nucleotides differing from the consensus sequence. The light chain repertoire was more diverse and included 7 distinct kappa and 3 distinct lambda light chains (Suppl. Table 1). While the common heavy chain of all clones shared the very same VDJ-gene segment usage as mAb FI6 [IGHV3-30, IGHD3-9, IGHJ4*01• 16], none of the light chains isolated in this experiment displayed the same IGKV4-1xIGKJ1 genotype of the FI6 light chain (Suppl. Table 1).

Since at that time, data on affinity maturation of FI6 was not yet available, we randomly selected a representative clone based on phage-ELISA data using H2 as coating antigen. This clone, referred to as mAb 3.1, expressed the common heavy chain paired to an IGKV1-12 x IGKJ4*01 light chain. Interestingly, sequencing revealed that the light chain present in mAb 3.1 hardly displayed any somatic hypermutation, and only displayed nucleotide replacements in FR1 causing aa 1 and 2 to differ from the reference alleles deposited at IMGT. However, these mutations are most likely an artifact arising from serial PCR amplification required for the preparation of a phage display library.

Specificity of mAb 3.1

In enzyme-linked immunosorbent assays (ELISA), mAb 3.1 was found to bind to recombinant HA proteins from A/Puerto Rico/8/1934(H1N1), and A/Japan/305/1957(H2N2), but failed to bind to A/Moscow/10/1999 (H3N2) and A/Fowl plague/Bratislava/1979(H7N7). The EC50 values were calculated to be $3x10^{-8}$ for H1, and $1.8x10^{-8}$ g/l for the H2, respectively (data not shown). Using Bio-layer interferometry (BLI), Fab fragments of mAb3.1 were also found to bind to biotinylated HA from A/duck/Alberta/345/1976(H1N1), A/USSR/90/1977(H1N1), A/Beijing/262/1995(H1N1), A/Solomon Islands/3/2006(H1N1), A/Japan/305/1957(H2N2), A/Adachi/2/1957(H2N2), A/Vietnam/1203/2004 (H5N1), A/turkey/Massachusetts/3740/1965(H6N2). No binding was neither found by ELISA nor BLI to A/duck/Alberta/60/1976(H12N5), A/gull/Maryland/704/1977(H13N6), A/black-headed gull/ Sweden/4/99 (H16N3), A/duck/Ukraine/1/1963 (H3N8), A/Hong Kong/1/1968 (H3N2), A/duck/Czechoslovakia/1956 (H4N6), A/Netherlands/219/2003 (H7N7), A/Fowl plague/Bratislava/1979 (H7N7), A/chicken/Germany/N/1949 (H10N7), A/mallard/Astrakhan/263/1982 (H14N5), A/shearwater/ W. Australia/2576/79 (H15N9) (Supplementary Table 2). These data show that mAb 3.1 bound HA proteins from phylogenetic group 1 but failed to bind HA proteins belonging to phylogenetic group 2.

The antiviral activity of mAb3.1 against at least one representative isolate from subtypes H1 through H15 was tested. Since pseudo-typed influenza viruses were described to be more easily neutralized than live virus [13], all of our neutralization assays were performed with viable influenza viruses. To this end, we established a robust fluorescence-based neutralization assay that employed 10^5 infectious units per well, corresponding to a multiplicity of infection of 2 to 3. Using this assay, it was found that mAb 3.1 neutralized viruses of the H1, H2, H5, H6 subtypes at half maximal inhibitory concentrations in the µg/ml range (Figure 1). Thus, like most V_H1-69-encoded and the germline-reverted variants of FI6, mAb 3.1 was only able to neutralize isolates from phylogenetic group 1. However, mAb 3.1 failed to neutralize isolates from the H1b clade that includes the H11, H13, and H16 subtypes, and it hardly neutralized isolates from the H9 clade (i.e. H8, H9, H12). The main differences in the stem epitope between the H1b and the remaining clades are a glycosylation site at position 291 of HA1, and the lack of a proline at position 293. As a consequence, interaction of HA1 with FR3 residues of mAb 3.1 may be disturbed. However, since the only exception with this respect, H6, is well recognized by mAb 3.1, these differences alone are unlikely to be the sole reason for the different recognition by mAb3.1. Indeed, removal of the glycosylation site at position 291 in the hemagglutinin of A/duck/Memphis/546/1974(H11N9) and reassortment into A/Puerto Rico/8/1934(H1N1) did not improve 3.1-mediatied neutralization sensitivity of the resulting reassortant virus (Figure 2). However, if the removal of the glycosylation site at position 291 was accompanied by the introduction of a Pro at position 293, the resulting virus became partially sensitive to neutralization by mAb3.1. In contrast, neither the introduction of a glycosylation site at position 291 or removal of the proline at position 293 of the hemagglutinin from A/Puerto Rico/8/1934(H1N1) altered its neutralization sensitivity. Thus, depending on the structural context, these two residues may have a great impact on the neutralization sensitivity to antibodies binding to this epitope.

In vivo protection

Encouraged by the neutralization data, we assessed whether passively transferred mAb 3.1 was able to protect mice from a lethal challenge with A/Puerto Rico/8/34(H1N1). As depicted in Figure 3, all mice were protected against a lethal challenge with 2 LD50 of A/Puerto Rico/8/34(H1N1) when 10mg/kg mAb3.1 were transfused 24h before infection. Also at 3mg/kg, 8 out of 10 mice were protected against the same dose of virus in two independent experiments (Figure 3). Weight loss in infected animals was considerably attenuated at 10mg/kg with 9 out 10 animals losing less than 15% of their initial body weight. Also at 3mg/kg, 7 out the 8 surviving mice lost less than 15% of the initial body weight during the course of the infection. In contrast, the weight of all control animals dropped below 15% already at day 4. Thus, the *in vivo* protective capacity of mAb3.1 was comparable to FI6,

another V_H3-30 encoded monoclonal antibody and in the same range as all other heterosubtypic antibodies described. In accordance to 3R recommendations of the Swiss animal welfare authorities, we therefore did not perform any further *in vivo* experiments with other strains, as we did not expect any new insights (or surprises) from a replication of these experiments.

Crystal structure of mAb 3.1

In order to analyze the molecular interaction with HA, we solved the crystal structure of the Fab fragment of mAb3.1 in complex with soluble HA from a pandemic H1N1 influenza virus A/South Carolina/1/18(H1N1) at 2.9 Å resolution (Figure 4). Not surprisingly, mAb31 recognizes the same hydrophobic groove in the stem as other V_H3-30 or V_H1-69-encoded antibodies. The epitope consists of residues from the N- and C-terminal regions of HA1 (38, 40-42, 289-293, 318), and the N-terminal portion of HA2 (18-21, 38, 41, 42, 45, 49, 52, 53, 56), including helix A. mAb3.1 buries a total of ~1333 $Å^2$ at the interface with HA (686 $Å^2$ for HA and 647 for Fab) and almost exclusively uses residues of the heavy chain (Fab3.1 heavy chain contributes 96% of the Fab buried surface area) for these interactions (Figure 4A, Supplementary Table 3). The angle of approach of Fab 3.1 is similar to murine antibody C179 and FI6, despite different binding interaction [12, 16]. The VH domain binds using a combination of 3 loops, including HCDRs 1 and 3, and the tip of FR3 (Figure 4B). HCDR1 and HCDR3 account for 85% of van der Waals contacts between Fab and HA, where Phe27 (HCDR1), Tyr99 and Phe100 (HCDR3) make many of the key hydrophobic interactions (~50% of van der Waals). Corresponding HCDR3 interactions were observed in the FI6 and C179-HA complexes, where both insert two aromatic side chains from their HCDR3 ($Tyr99^{mAb3.1}$, $Tyr98^{C179}$ and $Tyr100C^{FI6}$; and $Phe100^{mAb3.1}$, $Phe99^{C179}$ and $Phe100D^{FI6}$) into the hydrophobic groove (Figure 4B). As previously described antibodies (CR6261, F10, C179) or protein designed (HB36 and F-HB80.4) specific to group1 influenza A viruses, mAb3.1 make a similar interaction with Trp21 placing Phe100 at the tip of HCDR3 at a remarkably similar position and orientation (Figure 4A) [12-14, 16, 22, 23].

In addition to the HCDR-mediated interactions, residues 74 through 76 of framework region 3 contact Asn289 and Ser291 of HA1. Like FI6, mAb 3.1 also employs LCDR1 to contact HA2. However, in case of mAb3.1, these contacts do not involve the fusion peptide but are formed by van-der-Waals interactions of Gln38 located at the N-terminal end of the A helix with Trp 32 of the light chain. In contrast to FI6, where a long HCDR3 solely mediates contacts with the hydrophobic grove, mAb31 employs a combination of HCDR1 and H3 to contact HA protein (Figure 4B). Thereby the membrane proximal contacts are made by HCDR3, while the residues of HCDR1 provide the apical interactions.

Of note, it was found that the de novo *in silicio* designed and affinity matured artificial protein HB36.3 closely mimicked some of the interactions with mAb3.1. In particular, the α-helical structure of HCDR1 is reminiscent of HB36.3's recognition helix with very similar hydrophobic interactions mediated by Phe side chains at position 27 and 100 of mAb3.1 and position 49 and 61 of HB36.3 (Figure 4B). Comparison with the crystal structure of unbound mAb 3.1 revealed that twisting of HCDR1 Phe27 in the bound configuration is most likely to trigger this α-helix formation (Figure 4C). Structural homology search revealed that other V_H3-30-encoded monoclonal antibodies, such as 8F9 recognizing the neutralizing AD-2S1 epitope of the human cytomegalovirus glycoprotein B (gB)[24], B7-12A2 specific for tetanus toxoid [25], or APU2.16 recognizing polyubiquitin in a linkage-specific manner [26] also display α-helical HCDR1 structures, that can also be found amongst the canonical IgG structures [27]. Yet, the striking similarities with HB36.3 demonstrate nicely that *in silicio* designed and *in vitro* maturated artificial proteins can simulate naturally selected antibody binding pretty well.

In contrast to most other heterosubtypic antibodies where the light chain was described not to be essential for binding, FI6 was found to possess two residues (F27 and R93) in its light chain whose reversion to germline-encoded serine drastically reduced the ability to bind to HA proteins of phylogenetic group 2 HA, even when introduced individually [16]. However, when the FI6 light chain was paired with the heavy chain of 3.1, the hybrid antibody displayed the binding and neutralization profile of mAb3.1, as tested by binding to H1, H3, H7, H12 and neutralization of H3, H4, H7, H10, H12, or H15 isolates (data not shown). This indicates that the beneficial impact of these FI6 light chain residues is specific to FI6.

Discussion

We have isolated a V_H3-30 encoded heterosubtypic monoclonal antibody that neutralizes viruses from the H1a clade very well and displays low neutralizing activity against the H9 clade. mAb3.1 contacts a similar epitope in the stem of the HA as all other V_H3-30 and V_H1-69 encoded antibodies and primarily involves residues of the heavy chain, which bind to the hydrophobic groove in this epitope. Our finding, together with recently published heterosubtypic antibodies from an H1N1pdm-vaccinated donor [20], fortifies the predominance of V_H1-69 and V_H3-30-encoded antibodies in the human repertoire of heterosubtypic antibodies. In case of mAb3.1, only 4 out of 10 somatically hypermutated residues of the V_H gene are actually involved in the binding of the HA protein, suggesting that the majority of the binding residues are already satisfactorily encoded in the V_H3-30 germline gene. However, in contrast to FI6 that predominantly employs residues of HCDR3, mAb3.1 uses a combination of both HCDR1 and HCDR3 to contact residues of the hydrophobic groove. Thus, in both

V_H3-30 encoded heterosubtypic antibodies, diversity generated by recombination in the HCDR3 appears to be of great importance since in FI6 residues contacting the conserved epitope almost exclusively arise from the antibody gene rearrangement. But also in case of mAb3.1, a good proportion of the binding energy originates from hydrogen bonds or hydrophobic interactions of the residues of HCDR3. Quite interestingly, both V_H3-30-endcoded heterosubtypic antibodies required extensive addition of non-templated N-nucleotides. In case of mAb3.1, there is no addition of N1 nucleotides but the N2 region contains 17 nucleotides (5'-tcataaggggcattatg-3') encoding for 7 aa (N-FIRIGIM-C), two of which (F100, R100B) contribute to binding. In case of FI6, the N-nucleotide additions are even more extensive as there are a total of 32 non-templated nucleotides added, 22 nt of which as N1 (5'-c tcc caa ctg cga tca ctc ctc-3') and 10 as N2 (5'-cc cag gga tat-3') nucleotides that also contain key residues for heterosubtypic binding (L98, R99). Both antibodies use the D3-9*01 segment that provides these antibodies with two essential hydrophobic residues that are inserted into the groove. Thus, the V_H3-30 germline gene appears to provide good framework for heterosubtypic antibodies as it provides a HCDR1 that can (or cannot) be used to contact the apical region of the conserved stem epitope. However, this framework needs to be combined with D3-9, or another D region capable of providing hydrophobic residues, and these need to be sufficiently separated from the germline framework by the addition of N nucleotides to be inserted deep into the conserved hydrophobic grove in the stem of the HA protein. In contrast to V_H1-69 encoded antibodies, the light chain can play a supportive role and provide additional free energy for binding of HA. With regard to the contribution of the complementarity determining regions to the overall binding, HCDR1 and 3 using mAb 3.1 appears to be in between FI6 that primarily uses HCDR3 and V_H1-69 encoded antibodies that employ all three HCDRs for binding [13, 14, 19].

Of note, literature search revealed that the V_H3-30 and V_H3-11 germline genes are suspected to be evolutionary selected to encode for CMV-neutralizing antibodies, as most key residues required for binding of the linear neutralizing epitope AD-2S1 were already found in these germline genes [24, 28]. Indeed, structural homology searches using PDBe or DALI search engines identified cytomegalovirus neutralizing antibody 8F9 [28] (MJ5; PDB 3EYG), as the structurally most closely related antibody. However, a functional relationship would appear highly unlikely. Indeed, neither mAb3.1 nor FI6 displayed detectable binding to CMV glycoprotein B in ELISA, excluding a functional overlap between V_H3-30-encoded influenza and CMV-neutralizing antibodies.

It has been described that germline-reverted CMV-neutralizing antibodies are highly poly- and potentially autoreactive [29]. Similar findings have been made for an IGHV3-33-encoded HIV-neutralizing antibody [30]. It has been speculated that due to the potential autoreactivity of their germline antibodies, generation of certain antibody specificities would be more difficult. Accordingly,

the V_H3-30 germline gene would represent a weak spot in the antibody repertoire that is actively exploited by variable viruses. A recent germline-usage evaluation, however, demonstrated that V_H3-30-encoded antibodies are the fourth most frequently entered antibody sequences into the IMGT database (as of July 5, 2013). We therefore find it unlikely that a potential auto-reactivity of the germline-encoded antibodies can provide a satisfactory explanation for the rare occurrence of heterosubtypic antibodies.

So far, the antigenic particularity of the H1b clade has been underappreciated. As depicted in Figure 2, the C-terminal region of HA1 displays considerable differences in the H11, H13 and H16 subtypes compared to other members of phylogenetic group 1 (except H6). In particular, there is a potential N-linked glycosylation site at position 291 and no proline at position 293 in this clade. These differences in the proximity of the contacting FR3-residues may perturb binding of heterosubtypic antibodies. Indeed, even the broadest antibody described so far, CR9114 appears to be struggling with this clade as it failed to neutralize an H11 isolate despite binding decently to recombinant H13 and H16 HA protein in ELISA [19]. We could show that the removal of the glycosylation site by the introduction of a proline at position 293 made H11 more susceptible to neutralization by mAb 3.1. However, when the reciprocal mutations were introduced into H1, no effect was seen. No influence on the neutralizing activity of FI6 was observed either. Thus, although there is no universal importance of this region on binding of the heterosubtypic antibodies, its composition can have substantial impact on neutralization by certain antibodies.

Thus, isolation and characterization of mAb3.1 helped to gain a better insight into the molecular requirements for binding to the conserved epitope in the stem of the HA protein. Moreover, it provided evidence that binding of *in silicio* and *in vitro* selected artificial antibodies closely resembles that of naturally occurring antibodies. This increasing molecular knowledge about the conserved epitope in the stem of the HA protein will help to develop universal influenza vaccines directed to this epitope.

Methods

Viruses

For preparation of viral stocks MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 and and the resulting viruses were harvested 48 to 76 hours after infection. Alternatively, embryonated hen eggs were inoculated with titrated amounts of virus and incubated for 48h at 37°C

before the allantoic liquid was harvested. Virus-containing supernatant or allantoic liquid was stored in aliquots at -70°C.

Reassortant viruses

If not available, HA genes of interest were amplified and cloned into pHW2000 as described by Hoffman et al. [31] For the generation of reassortant viruses, a pHW2000-derived plasmid containing segment 4 was mixed with plasmids containing the remaining 7 genome segments from A/Puerto Rico/8/1934(H1N1), and transfected into a mixture of 293T and MDCK cells as described by [32]. Three days after transfection, presence of virus was determined by hemagglutination and clarified supernatant was used to infect MDCK cells. Three days later, P1 supernatant was harvested and frozen at -70°C

Library construction and phage display selection of cross-reactive Fab clones

The antibody phage display library was prepared according to Barbas et al. [33]. In brief, donor 13 (RI-13), a healthy Caucasian male of 32 years was selected for this study due to slightly elevated titers against recombinant H5 protein in a preliminary screening ELISA. According to the questionnaire filled at the time of the blood draft, donor 13 had been vaccinated 6 times against seasonal influenza, had not knowingly been exposed to avian influenza A viruses, and did not experience an influenza episode or vaccination during the 3 months prior to the blood donation (April 4th 2009). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-cushion centrifugation on the day of the draft, and cells were cryopreserved in aliquots of $2x10^7$ PBMCs for later use.

At the day of the library preparation, ~$1.6x10^6$ mature B cells were isolated from thawed PBMCs using anti CD22-coated MACS beads (Milteny Biotech) according to the manufacturer's instructions. Total RNA was isolated from B cells using RNeasy Mini Kit columns (Qiagen) and was reverse transcribed into cDNA using Superscript II reverse transcriptase from Invitrogen and oligo dT primers (Promega) according to the manufacturer's recommendations. Immunoglobulin variable regions were PCR amplified from this cDNA, and assembled into Fab fragments in 3 subsequent PC reactions according to [33]. Ligation of the assembled Fab fragments into the pComb3X phage display vector yielded $1.5x10^9$ plasmid clones that gave rise to more than 10^{13} plaque forming units after super-infection with a helper phage.

For the panning, biotinylated and trypsin-digested hemagglutinin from A/Japan/1957(H2N2) was immobilized on streptavidin coated magnetic beads (Promega). Approximately $2.5x10^{12}$ of phag-

es were combined with 15µg of HA immobilized on 300 µl magnetic beads (final concentration of immobilized HA was 100 nM) for the first round of selection. A total of 4 panning rounds were conducted with increasing the stringency by using less protein-coated beads (2 µg of HA immobilized on 50 µl magnetic beads) and increasing the number of washes (1st round: 2x TBST; 2nd round: 4x TBST, 1xTBS; 3rd round: 6x TBST, 1xTBS; 4th round: 8xTBST, 1xTBS; TBST corresponds to TBS supplemented with 0.05% Tween 20). Phage clones obtained after the 3rd and 4th round were screened for binding to various HAs in ELISA, and positive clones were sequenced. One clone, referred to 3.1 was chosen for further analysis based on its sequence and binding properties.

Expression and purification of recombinant HAs

Recombinant HA, stabilized by a his-tagged trimerization domain, was expressed into the supernatant of baculovirus-infected SF9 insect cells as previously described [34]. After 4 days, supernatant was harvested and soluble protein purified by metal affinity chromatography (NiNTA columns, GE Healthcare). Purified HA was proteolytically processed into its HA1 and HA2 subunits using 10U of TPCK-treated trypsin (from bovine pancrease, Sigma Aldrich) per 1 µg of HA for 1h at RT. Following digestion, trypsin was removed by size exclusion chromatography using a 200ml Superdex® S200 gel filtration column (GE Healthcare). For further experiments, only the fraction corresponding to the HA trimer was used.

Expression and purification of recombinant Fab or IgG1 molecules.

For the purification of Fab 3.1 the protocol by Barbas et al. was followed [33]. Briefly, the phagemid containing the 3.1 sequence was transformed into chemically competent TOP 10 E. coli cells (Invitrogen). A single colony from the transformation plate was inoculated into LB supplemented with carbenicilin (50 µg/ml), and grown under agitation (200 rpm) at 37^{o}C over night. This pre-culture was then diluted 1:100 in SB supplemented with carbenicilin (50 µg/ml) and 20 mM MgCl2, and was grown under agitation (250 rpm) at 37^{o}C for 24h. Bacterial cells were harvested by centrifugation and disrupted using a sonicator (Branson Sonifier 250). Lysate was cleared by centrifugation (>13500 rpm for 60min) and filtration at 0.2 µm, and Fab fragments isolated by affinity chromatography using protein G slury (GE healthcare). Bound Fab was eluted from the column using 0.1 M glycine pH 3, and stored in PBS at 4°C after buffer exchange.

For expression of soluble IgG 3.1, FI6 and FI6-3.1 hybrid the variable regions of heavy and light chains were cloned into the corresponding pIg-Abvec plasmids [35]. Proteins were expressed by transient transfection of 293T cells (30 µg of each plasmid combined with 120 µg PEI per 1 T150 flask).

Cell supernatant was harvested, spinned down first at 3000 rpm 5 min and next at 8000 rpm 10 min, filtered at 0.2 µM, and recombinant IgG1 was purified by affinity chromatography using protein G columns. Eluted IgG1 was re-buffered into PBS and stored at 4°C.

ELISA

Binding of IgG 3.1, FI6 and FI6-3.1 to recombinant HA proteins (non trypsin digested H1 from A/Puerto Rico/8/34 (H1N1) and H7 from A/FPV/Bratislava/79 (H7N7), trypsin-digested and non-digested H3 from A/Moscow/10/99 (H3N2), and trypsin-digested H12 from A/Duck/Alberta/60/76 (H12N5) was detected by ELISA. To this end, half-area, high binding capacity plates (Costar) were coated with 25 µl/well of 2 µg/ml HA in PBS at 4 ^{o}C over night. Plates were then blocked with 2% milk in PBS. IgG were titrated in 0.2% milk PBS, transferred to the blocked ELISA plates, and allowed to bind for 1 h. After washing with TBST (0.1% Tween), bound Ig was detected using a goat anti-human kappa-HRP secondary antibody (Southern Biotech) and developed using TMB as a substrate. As a negative control, HIV gp120-specific and 293T-cell expressed mAb b12 antibody was used

Neutralization of Influenza A viruses

Titrated amounts of IgG 3.1, or IgG_1-b12 as negative control, were mixed in triplicates with a fixed amount of Influenza A virus corresponding to MOI 2-3 (~ 10^5 pfu) in DMEM medium supplemented with 0.2% BSA, 20 mM HEPES, 50U/ml penicillin and 50µg/ml streptomycin (DMEM/BSA). After incubation at 37^{o}C/5% CO_2 for 2h, the mAb-virus mixture was transferred to PBS washed, sub-confluent MDCK cells seeded into 96-well tissue culture plates the day before ($1.5x10^2$ cells/ well, TPP), and incubated at 37^{o}C/5% CO_2 for 1h to allow infection. Residual virus and antibody was aspirated, cells washed with PBS, and DMEM/BSA was added. Following incubation at 37°C/5% CO_2 for 4.5 to 7h (depending on the growth kinetics of the virus isolate), cells were fixed with methanol, washed and stained with a 3 µg/ml FITC-labeled antibody to influenza NP (ATCC HB-65™) in PBS containing 1% BSA at 4°C over night. After washing the FITC-labeled antibody, cells were stained with DAPI to control for cell density or cell loss. The corresponding fluorescence was then measured in each well at 16 (FITC) and 9 (DAPI) distinct locations points in a Perkin Elmer plate reader. For each well, the average for all individual fluorescence measuring points was calculated and used for further analysis. EC50 values were determined in Prism 5 (GraphPad Software) using iterative computing of the best fitting Hill equation.

Infectivity reduction assay

40 µl of DMEM/BSA containing 60 µg/ml of the antibody of interest were mixed with 80µl of untitrated virus supernatant and incubated for 90min at 37°C/CO_2. As a control, the same amount of virus was mock-incubated with DMEM/BSA without antibody. Following incubation for 90min at 37°/ CO_2, non-neutralized infectivity was determined by serial diluting the virus/antibody mixture 1 in 2, and infection of 2-4x10^4 MDCK cells with this dilution series. Infection was allowed to proceed 5-6 hours before cells were fixed and stained with a FITC-labeled antibody to NP, as described above.

Structural homology search

To identify the closest structural homologs of mAb 3.1, the coordinates for the 3.1 antibody heavy and light chain was extracted from the structure of the complex and submitted to the PDBe (http://www.ebi.ac.uk/msd-srv/ssm/ssmstart.html) or DALI (http://ekhidna.biocenter.helsinki.fi/dali_server/) structural homology search engines as a pdb file.

Kd Determination

Kd values were determined by bio-layer interferometry (BLI) using an Octet Red instrument (ForteBio, Inc.) as described in [19]. Biotinylated HAs were used for these measurements. HAs at ~10-50 µg/mL in 1X kinetics buffer (1X PBS, pH 7.4, 0.01% BSA, and 0.002% Tween 20) were loaded onto streptavidin-coated biosensors and incubated with varying concentrations of mAb3.1 Fab. If no initial binding was observed using the above conditions, mAb3.1 concentrations up to 1uM were used to detect whether changes could be observed in the binding curves.

Crystallization and structure determination of Fab3.1-Sc1918/H1 HA

For Fab/HA complex formation, mAb3.1 Fab was added to Sc1918/H1 HA in a molar ratio of ~3.2:1 to saturate all of the mAb3.1 binding sites on the HA trimer. The mixture was incubated over-night at 4°C to allow complex formation. Saturated complexes were then purified from unbound Fab by gel filtration and concentrated to ~10 mg/mL in 10mM Tris-HCl, pH 8.0 and 50 mM NaCl. Fab3.1-Sc1918/H1 HA crystals were grown by sitting drop vapor diffusion at 20°C by mixing 0.5 µL of concen-trated protein sample with 0.5 µL of mother liquor (15% PEG 3350, 0.1M sulfate de Magnesium, 100mM Tris-HCl pH7.5) and crystals appeared after 3 days. The resulting crystals were cryoprotected by soaking the crystals in well solution supplemented with increasing concentrations of ethylene

glycol (5% steps, 5 min/step), to a final concentration of 35%, then flash cooled and stored in liquid nitrogen.

Diffraction data were collected at the Canadian Light Source (CLS). The data were indexed in space group R3, scaled and integrated using Denzo and Scalepack through the HKL2000 package (HKL Research). Detailed data collection and refinement statistics are summarized in Supplementary Table 4. The structure was solved by molecular replacement to 2.9 Å resolution using Phaser [36]. Rigid body refinement, simulated annealing and restrained refinement (including TLS refinement, one for each Ig domain) were carried out in Refmac [37]. Between rounds of refinement, the model was re-built and adjusted using Coot [38].

Crystallization and structure determination of mAb3.1 Fab

The methods used to determine the mAb3.1 Fab structure were very similar to those described above. Briefly, mAb3.1 Fab at 15 mg/ml in 10mM Tris, pH 8.0 and 50 mM NaCl was subjected, after gel filtration, to robotic crystallization trials using the Rigaku Crystalmation robotic system at the Joint Center for Structural Genomics (JCSG). Several hits were obtained. The crystals used for data collection were grown by the sitting drop vapor diffusion method with a reservoir solution (1 mL) containing 0.2 M calcium acetate, 10% PEG 8000 and 100 mM Tris pH7.0. The resulting crystals were cryoprotected by soaking in well solution supplemented with 35% ethylene glycol, then flash cooled and stored in liquid nitrogen until data collection.

The mAb3.1 dataset was collected to 2.7 Å resolution at APS GM/CA-CAT 23ID-B beamline. Detailed data collection and refinement statistics are summarized in Supplementary Table 4. The structure was solved using the same strategies as described for the Fab3.1-Sc1918/H1 complex.

Structural analysis

Hydrogen bonds and van der Waals' contacts between mAB3.1 Fab and Sc1918/H1 HA were calculated using HBPLUS and CONTACSYM, respectively [39, 40]. Surface area buried upon Fab binding was calculated with MS [41]. MacPyMol (DeLano Scientific) was used to render structure figures and for general manipulations. Kabat numbering was applied to the coordinates using the Abnum server [42]. The final coordinates were validated using the JCSG quality control server (v2.7), which includes Molprobity [43].

Figures

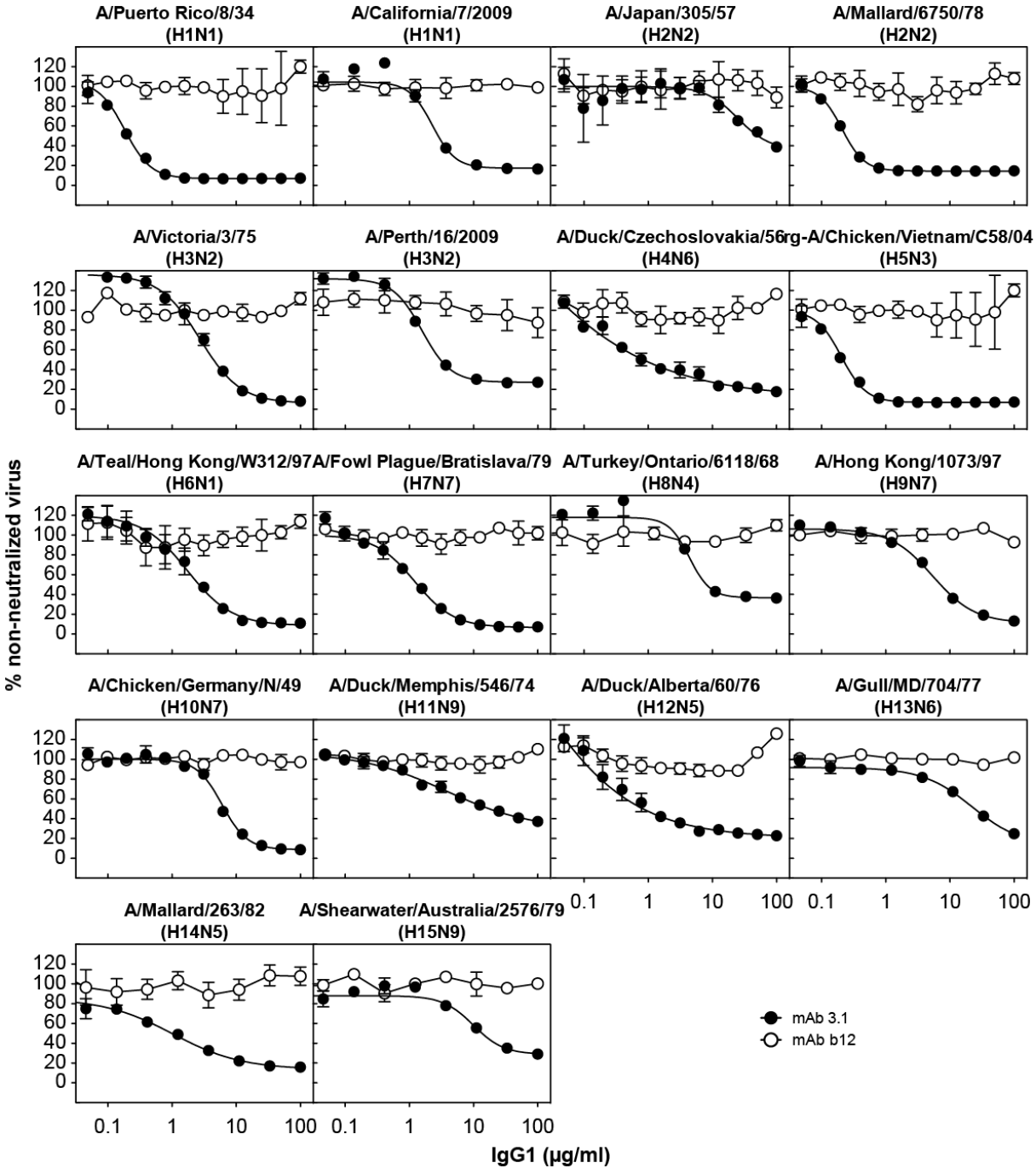
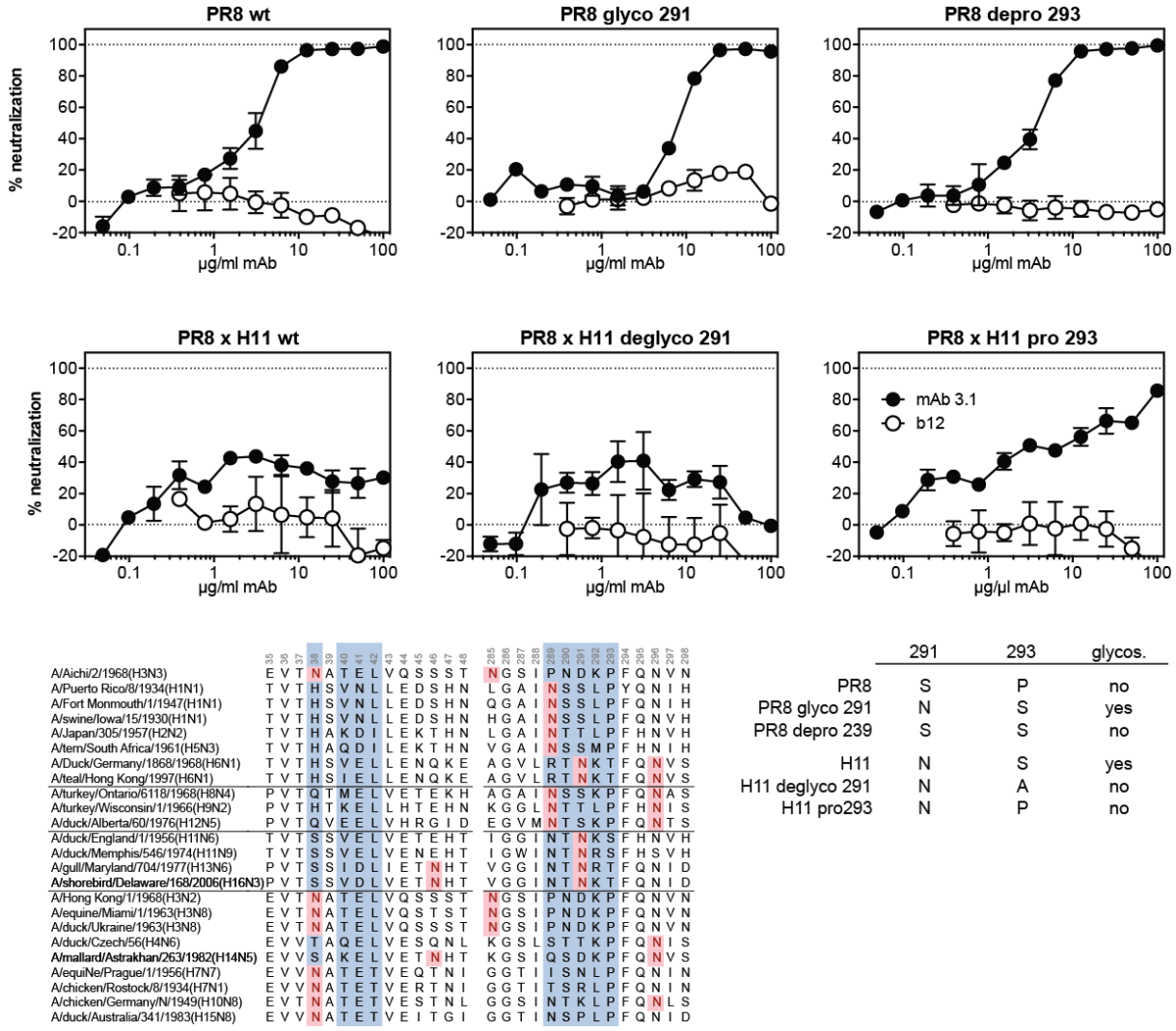


Figure 1: Virus neutralizing activity of mAb 3.1



	291	293	glycos.
PR8	S	P	no
PR8 glyco 291	N	S	yes
PR8 depro 239	S	S	no
H11	N	S	yes
H11 deglyco 291	N	A	no
H11 pro293	N	P	no

Figure 2: Neutralization sensitivity of wt and reassorted A/Puerto Rico/8/1934(H1N1) carrying wt or mutant H11 hemagglutinin from A/duck/Memphis/546/1974 (H11N9). To remove glycosylation at position 291, aa 293 was mutated from threonine to alanine (deglyco 291) or prolin (pro 293). The reciprocal mutations have been introduced into H1 as indicated in the table.

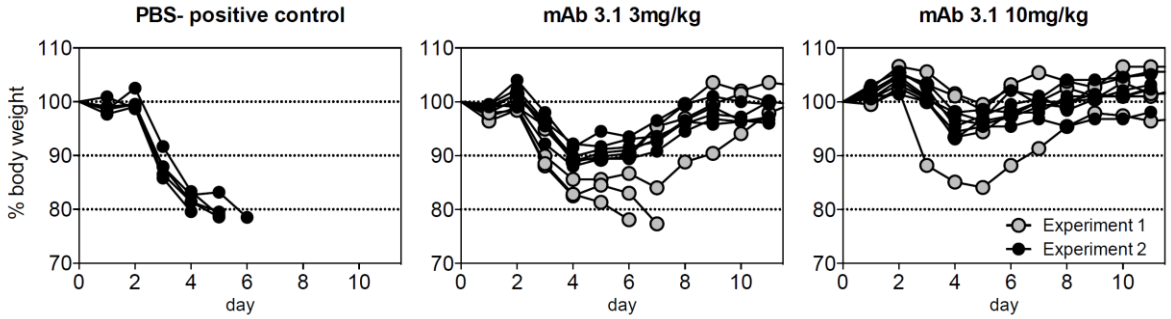


Figure 3: In vivo protection by passive immunization with mAb 3.1. The indicated dose of mAb or PBS were injected i.p. 24h before intranasal infection with 2xLD50 of A/Puerto Rico/8/34(H1N1). Body weight was monitored and mice were taken out of the experiment when their weight dropped below 80% of the initial body weight. Data shown were pooled from two independent experiments.

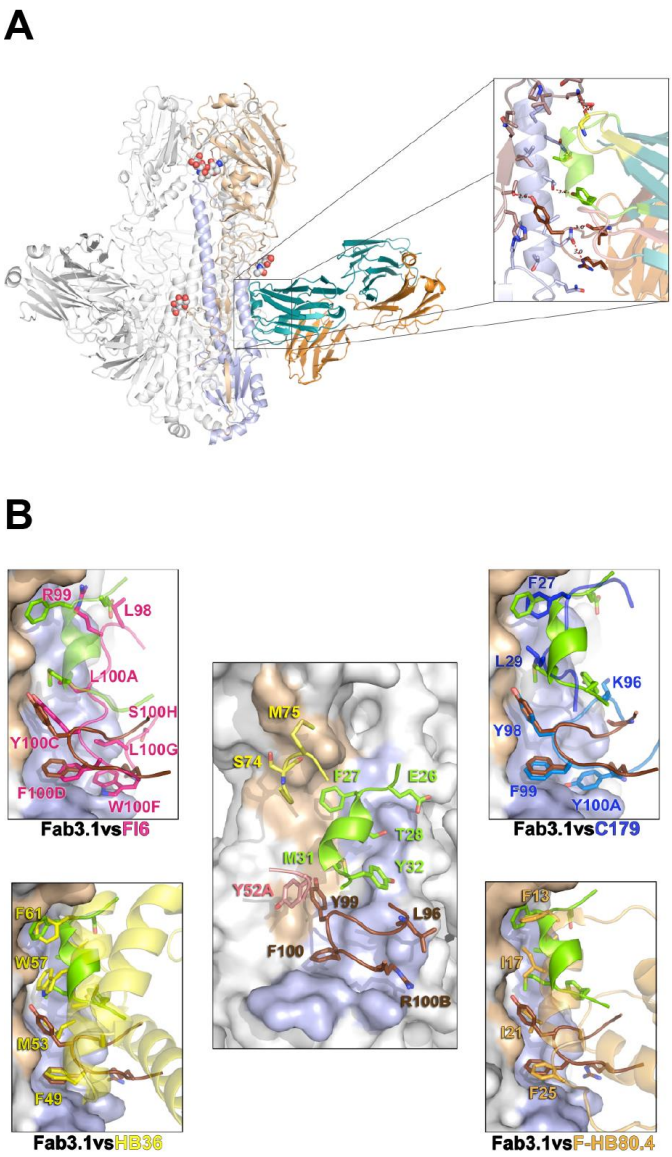


Figure 4: Crystal structure of Fab 3.1 bound to HA from A/South Carolina/1/1918(H1N1) **(A)** Overview of the antibody binding to the conserved epitope in the stem of the HA protein. **(B)** Comparison of epitope recognition of Fab 3.1 with mAbs FI6 and C179, as well as artificially binding proteins HB38, and F-HB80.4. The interacting residues on HA1 were colored light brown and on HA2 violet. **(C)** Comparison of CDRH1 free (gray) and in complex with HA from A/South Carolina/1/1918(H1N1).

Supplementary Tables

mAb	VH	DH	JH	CDR3H	VL	JL	CDR3L
3.1	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV1-12*01, or IGKV1-12*02 or IGKV1D-12*02	IGKJ4*01	CQQANSFPLTF
3.2	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ3*02	CSSHTSSSTWVF
3.4	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV6-57*01	IGLJ7*01	CQSYDNLNHAVF
3.5	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF
3.7	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV3-21*01	IGLJ2*01, or IGLJ3*01	CQVWDSHGDQVVF
3.8	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-11*01	IGKJ3*01	CQQRSNWPVTF
3.9	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV1-44*01	n.a.	n.a.
3.10	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQHYGASPKTF
3.11	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV1-44*01	IGLJ3*02	CSSWDGGLSDWVF
3.12	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3D-20*01	IGKJ1*01	CQQYGSSPQTF
3.13	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV1-33*01, or IGKV1D-33*01	IGKJ4*01	CQQHDNLPLTF
3.14	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ2*01	CQQYGGSPPYTF
3.15	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV6-57*01	IGLJ3*02	CQSYDSSNQWVF
3.16	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ2*01, or IGLJ3*01	CSSYTSSSTVVF
3.17	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ4*01	CQQYGSSPLTF
3.18	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGLV2-14*01	IGLJ2*01, or IGLJ3*01	CSSYTSSSTVVF
3.48	IGHV3-30*04	IGHD3-9*01	IGHJ4*01, or IGHJ4*03	CARDLGGYFIRGIMDVW	IGKV3-20*01	IGKJ1*01	CQQYGSSPRTF
FI6	IGHV3-30*03 F, or IGHV3-30*18 F	IGHD3-9*01	IGHJ4*02	CAKDSQLRSLLYFEWLSQGYFDPW	IGKV4-1*01	IGKJ1*01	CQQHYRTPPTF

Suppl. Table 1: Genetic hallmarks of the antibodies isolated by panning a phage display library against recombinant H2 protein, and FI6 for reference. Assignment of the germline genes and CDRs were performed using the vquest tools provided at IMGT (http://imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg)

Grp	Sub-type	Strain	Muta-tion	Kd (nM)	HA1						HA2																			
					38	*40*	*41*	*42*	*289*	*290*	*291*	*292*	*293*	*318*	*18*	*19*	*20*	*21*	*38*	*41*	*42*	*45*	*46*	*48*	*49*	*52*	*53*	*56*
1	H1N1	A/South Carolina/1/1918	wt	1	H	V	N	L	N	S	S	L	P	T	I	D	G	W	Q	T	Q	I	D	I	T	V	N	I
1	H1N1	A/duck/Alberta/345/1976	wt	1	H	V	N	L	N	S	S	L	P	I	I	D	G	W	Q	T	Q	I	D	I	T	V	N	I
1	H1N1	A/USSR/90/1977	wt	2.2	H	V	N	L	N	S	S	L	P	T	I	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H1N1	A/Beijing/262/1995	wt	0.9	H	V	N	L	N	S	S	L	P	T	M	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H1N1	A/Solomon Islands/3/2006	wt	2.8	H	V	N	L	N	S	S	L	P	T	V	D	G	W	Q	T	Q	I	N	I	T	V	N	I
1	H2N2	A/Japan/305/1957	wt	9.9	H	K	D	I	N	T	T	L	P	T	V	D	G	W	K	T	Q	F	D	I	T	V	N	I
1	H2N2	A/Adachi/2/1957	wt	10	H	K	D	I	N	T	T	L	P	T	V	D	G	W	K	T	Q	F	D	I	T	V	N	I
1	H5N1	A/Vietnam/1203/2004	wt	2	H	Q	D	I	N	S	D	M	P	T	V	D	G	W	K	T	Q	I	D	V	T	V	N	I
1	H6N2	A/turkey/Massachusetts/3740/1965	wt	25	H	V	E	L	K	T	N	K	T	T	I	D	G	W	K	T	Q	I	D	I	T	V	N	I
1	H12N5	A/duck/Alberta/60/1976		N.B	Q	E		L			S	K	P	T	V	A	G	W	R	T	Q	I	D		L		I
1	H13N6	A/gull/Maryland/704/1977		N.B	S	I		L			N	R	T	T	I	N	G	W	K	T	Q	I	D		I		I
1	H16N3	A/black-headed gull/Sweden/4/99		N.B	S	I		L			N	K	T	T	I	N	G	W	K	T	Q	I	N		I		I
2	H3N8	A/duck/Ukraine/1/1963		N.B	N	T		L			D	K	P	T	I	D	G	W	L	T	Q	I	D		L		I
2	H3N2	A/Hong Kong/1/1968		N.B	N	T		L			D	K	P	T	I	D	G	W	L	T	Q	I	D		L		I
2	H4N6	A/duck/Czechoslovakia/1956		N.B	T	Q		L			T	K	P	T	I	D	G	W	L	T	Q	I	D		L		I
2	H7N7	A/Netherlands/219/2003		N.B	N	T		T			N	L	P	T	I	D	G	W	Y	T	Q	I	D		L		I
2	H10N7	A/chicken/Germany/N/1949		N.B	N	T		T			K	L	P	T	V	D	G	W	Y	T	Q	I	D		L		I
2	H14N5	A/mallard/Astrakhan/263/1982		N.B	S	K		L			D	K	P	T	I	D	G	W	L	T	Q	I	D		L		I
2	H15N9	A/shearwater/W. Australia/2576/79		N.B	N	T		T			P	L	P	L	I	D	G	W	Y	T	Q	I	D		L		I

Suppl. Table 2: Binding of Fab 3.1 to HA from the indicated subtype. N.B.- Not Binding.

type of inter-action	Chain	Residue	Res. #	Atom	Chain	Residue	Res. #	Atom	# of int.	Dist.
VDW	HA1	HIS	38	CB	heavy	TYR	99	OH	1	3.56
VDW	HA1	HIS	38	CB	heavy	TYR	99	CZ	1	3.58
VDW	HA1	HIS	38	CB	heavy	TYR	99	CE1	1	3.9
VDW	HA1	HIS	38	CB	heavy	TYR	99	CE2	1	4.02
VDW	HA1	HIS	38	CG	heavy	TYR	99	CZ	1	3.94
VDW	HA1	HIS	38	CG	heavy	TYR	99	CE1	1	3.97
VDW	HA1	HIS	38	ND1	heavy	TYR	52A	OH	1	3.52
VDW	HA1	HIS	38	ND1	heavy	TYR	99	CD2	1	3.74
VDW	HA1	HIS	38	ND1	heavy	TYR	99	CE2	1	3.83
VDW	HA1	HIS	38	CE1	heavy	TYR	52A	OH	1	3.73
VDW	HA1	HIS	38	CE1	heavy	TYR	99	CG	1	4.1
VDW	HA1	VAL	40	CB	heavy	PHE	27	CE2	1	3.63
VDW	HA1	VAL	40	CB	heavy	PHE	27	CZ	1	3.96
VDW	HA1	VAL	40	CG1	heavy	PHE	27	CE2	1	3.94
VDW	HA1	VAL	40	CG1	heavy	MET	31	CG	1	4
VDW	HA1	VAL	40	CG1	heavy	MET	31	SD	1	4.15
VDW	HA1	VAL	40	CG2	heavy	PHE	27	CE2	1	3.91
VDW	HA1	ASN	41	O	heavy	PHE	27	CZ	1	3.33
VDW	HA1	ASN	41	O	heavy	PHE	27	CE1	1	3.73
VDW	HA1	LEU	42	CD2	heavy	PHE	27	CZ	1	3.77
VDW	HA1	LEU	42	CD2	heavy	PHE	27	CE2	1	3.9
SHORTVDW	HA1	ASN	289	OD1	heavy	MET	75	SD	1	3
VDW	HA1	ASN	289	OD1	heavy	MET	75	CG	1	3.81
VDW	HA1	SER	290	N	heavy	MET	75	CE	1	3.96
VDW	HA1	SER	290	CA	heavy	MET	75	CE	1	3.99
VDW	HA1	SER	290	C	heavy	MET	75	CE	1	3.71
VDW	HA1	SER	290	O	heavy	MET	75	CE	1	3.88
H-BOND	HA1	SER	291	N	heavy	SER	74	O	1	3.03
VDW	HA1	SER	291	CA	heavy	SER	74	O	1	3.65
SHORTVDW	HA1	SER	291	CB	heavy	SER	74	O	1	3.1
VDW	HA1	SER	291	CB	heavy	ARG	30	NH2	1	3.69
VDW	HA1	SER	291	CB	heavy	ASN	76	CB	1	3.86
H-BOND	HA1	SER	291	OG	heavy	SER	74	O	1	2.93
H-BOND	HA1	SER	291	OG	heavy	MET	75	O	1	3.12
VDW	HA1	SER	291	OG	heavy	MET	75	C	1	3.12
VDW	HA1	SER	291	OG	heavy	ASN	76	CB	1	3.37
VDW	HA1	SER	291	OG	heavy	MET	75	CE	1	3.37
VDW	HA1	SER	291	OG	heavy	MET	75	CA	1	3.51
VDW	HA1	SER	291	OG	heavy	ASN	76	N	1	3.58
VDW	HA1	SER	291	OG	heavy	SER	74	C	1	3.88
VDW	HA1	LEU	292	CD2	heavy	ARG	30	NH2	1	3.48
VDW	HA1	LEU	292	CD2	heavy	PHE	27	CE1	1	3.64
VDW	HA1	PRO	293	CD	heavy	PHE	27	CE1	1	3.9
VDW	HA1	PRO	293	CD	heavy	PHE	27	CD1	1	3.97
VDW	HA1	THR	318	CB	heavy	TYR	99	OH	1	3.43
H-BOND	HA1	THR	318	OG1	heavy	TYR	99	OH	1	2.63
VDW	HA1	THR	318	OG1	heavy	TYR	99	CZ	1	3.6
VDW	HA1	THR	318	OG1	heavy	TYR	99	CE1	1	3.87
VDW	HA1	THR	318	CG2	heavy	TYR	99	OH	1	3.88
VDW	HA1	THR	318	CG2	heavy	MET	31	CE	1	4.01
VDW	HA1	THR	318	CG2	heavy	MET	31	SD	1	4.04
VDW	HA2	ILE	18	O	heavy	PHE	100	CD1	1	3.46
VDW	HA2	ILE	18	O	heavy	PHE	100	CE1	1	3.56
VDW	HA2	ASP	19	C	heavy	PHE	100	CD1	1	3.67

VDW	HA2	ASP	19	O	heavy	PHE	100	CB	1	3.65
VDW	HA2	ASP	19	O	heavy	PHE	100	CG	1	3.74
VDW	HA2	ASP	19	O	heavy	PHE	100	CD1	1	3.81
VDW	HA2	GLY	20	N	heavy	PHE	100	CD1	1	3.55
VDW	HA2	GLY	20	N	heavy	PHE	100	CE1	1	3.69
VDW	HA2	GLY	20	CA	heavy	PHE	100	CE1	1	3.68
VDW	HA2	GLY	20	CA	heavy	PHE	100	CD1	1	3.7
VDW	HA2	GLY	20	CA	heavy	PHE	100	CZ	1	3.83
VDW	HA2	GLY	20	CA	heavy	PHE	100	CG	1	3.85
VDW	HA2	GLY	20	CA	heavy	PHE	100	CE2	1	3.99
VDW	HA2	GLY	20	CA	heavy	PHE	100	CD2	1	4.01
VDW	HA2	GLY	20	C	heavy	PHE	100	CZ	1	3.59
VDW	HA2	GLY	20	C	heavy	PHE	100	CE1	1	3.79
VDW	HA2	GLY	20	C	heavy	PHE	100	CE2	1	3.96
VDW	HA2	GLY	20	O	heavy	PHE	100	CZ	1	3.76
VDW	HA2	GLY	20	O	heavy	PHE	100	CE1	1	3.79
VDW	HA2	TRP	21	N	heavy	PHE	100	CZ	1	3.9
VDW	HA2	TRP	21	CG	heavy	PHE	100	CE2	1	3.85
VDW	HA2	TRP	21	CG	heavy	PHE	100	CZ	1	4
VDW	HA2	TRP	21	CD1	heavy	PHE	100	CZ	1	3.53
VDW	HA2	TRP	21	CD1	heavy	PHE	100	CE2	1	3.79
VDW	HA2	TRP	21	NE1	heavy	PHE	100	CZ	1	3.72
VDW	HA2	TRP	21	NE1	heavy	PHE	100	CE2	1	3.86
VDW	HA2	TRP	21	CE2	heavy	PHE	100	CE2	1	3.96
VDW	HA2	TRP	21	CD2	heavy	PHE	100	CE2	1	3.95
VDW	HA2	TRP	21	CH2	heavy	TYR	99	CE1	1	4.12
VDW	HA2	TRP	21	CH2	heavy	TYR	99	CD1	1	4.17
VDW	HA2	TRP	21	CZ2	heavy	TYR	99	CD1	1	3.66
VDW	HA2	TRP	21	CZ2	heavy	TYR	99	CE1	1	3.69
VDW	HA2	GLN	38	CB	heavy	ARG	100B	NH2	1	3.57
VDW	HA2	GLN	38	OE1	heavy	ARG	100B	NH1	1	3.6
VDW	HA2	GLN	38	OE1	light	TRP	32	CH2	1	3.64
VDW	HA2	GLN	38	OE1	light	TRP	32	CZ3	1	3.65
VDW	HA2	GLN	38	NE2	heavy	ARG	100B	CG	1	3.9
VDW	HA2	THR	41	CG2	heavy	PHE	100	CD2	1	3.7
VDW	HA2	THR	41	CG2	heavy	PHE	100	CE2	1	3.84
VDW	HA2	GLN	42	CD	heavy	LEU	96	O	1	3.65
H-BOND	HA2	GLN	42	OE1	heavy	ARG	100B	NE	1	2.96
VDW	HA2	GLN	42	OE1	heavy	ARG	100B	CD	1	3.35
VDW	HA2	GLN	42	OE1	heavy	LEU	96	O	1	3.54
VDW	HA2	GLN	42	OE1	heavy	GLY	97	CA	1	3.62
H-BOND	HA2	GLN	42	NE2	heavy	LEU	96	O	1	3.08
VDW	HA2	ILE	45	CG1	heavy	GLY	97	O	1	3.83
VDW	HA2	ILE	45	CD1	heavy	PHE	100	CD2	1	3.7
VDW	HA2	ILE	45	CD1	heavy	PHE	100	CE2	1	3.83
VDW	HA2	ILE	45	CG2	heavy	GLY	97	O	1	3.91
VDW	HA2	ILE	45	CG2	heavy	TYR	32	OH	1	4.03
VDW	HA2	THR	49	CA	heavy	THR	28	CG2	1	4.06
VDW	HA2	THR	49	CB	heavy	TYR	32	OH	1	3.55
VDW	HA2	THR	49	CB	heavy	THR	28	CG2	1	4.17
H-BOND	HA2	THR	49	OG1	heavy	TYR	32	OH	1	3.22
SHORTVDW	HA2	THR	49	CG2	heavy	TYR	32	OH	1	3.24
SHORTVDW	HA2	THR	49	CG2	heavy	MET	31	CE	1	3.42
VDW	HA2	THR	49	CG2	heavy	TYR	32	CE2	1	3.56
VDW	HA2	THR	49	CG2	heavy	TYR	32	CZ	1	3.79
VDW	HA2	THR	49	CG2	heavy	MET	31	SD	1	3.84

VDW	HA2	THR	49	CG2	heavy	THR	28	CB	1	3.93
VDW	HA2	THR	49	CG2	heavy	THR	28	CG2	1	3.98
VDW	HA2	THR	49	C	heavy	THR	28	CG2	1	4.13
VDW	HA2	THR	49	O	heavy	THR	28	CG2	1	3.4
VDW	HA2	VAL	52	CB	heavy	THR	28	CG2	1	3.8
VDW	HA2	VAL	52	CG1	heavy	THR	28	CG2	1	3.58
VDW	HA2	VAL	52	CG1	heavy	PHE	27	CD2	1	3.97
VDW	HA2	ASN	53	CG	heavy	THR	28	OG1	1	3.26
VDW	HA2	ASN	53	CG	heavy	THR	28	CG2	1	3.45
VDW	HA2	ASN	53	CG	heavy	THR	28	CB	1	3.97
H-BOND	HA2	ASN	53	OD1	heavy	THR	28	OG1	1	3.09
SHORTVDW	HA2	ASN	53	OD1	heavy	THR	28	CG2	1	3.15
VDW	HA2	ASN	53	OD1	heavy	THR	28	CB	1	3.63
H-BOND	HA2	ASN	53	ND2	heavy	THR	28	OG1	1	2.72
H-BOND	HA2	ASN	53	ND2	heavy	GLU	26	OE1	1	3.3
VDW	HA2	ASN	53	ND2	heavy	THR	28	CG2	1	3.55
VDW	HA2	ASN	53	ND2	heavy	THR	28	CB	1	3.69
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CD2	1	3.6
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CG	1	3.64
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CB	1	3.82
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CE2	1	4.21
VDW	HA2	ILE	56	CD1	heavy	PHE	27	CD1	1	4.27

Suppl. Table 3: Interactions of mAb 3.1 with HA from A/South Carolina/1/1918(H1N1). For graphic representation see Figure 4.

Data collection	Fab 3.1	Fab 3.1- SC1918/H1 HA
Beamline	APS GM/CA CAT 23ID-B	CLS
Wavelength (Å)	0.71941	0.97549
Space group	$P4_3$	R_3
Unit cell parameters (Å, °)	a =73.8, b =73.8, c = 207.9 α=β=γ=90	a =135.0, b =135.0, c = 230.2 α=β=90, γ=120
Resolution (Å) [a]	50 -2.7 (2.75- 2.70)	50-2.9 (2.95-2.9)
Observations	118,373	196,824
Unique reflections	30,297 (1515) [a]	34347 (1706)
Redundancy	3.9 (3.8) [a]	5.7 (4.7)
Completeness (%)	98.2 (98.7) [a]	99.6 (96.3)
$\langle I/\sigma_I \rangle$	12.2 (1.5) [a]	31.4 (2.1)
R_{sym} [b]	0.12 (0.65) [a, b]	0.1 (0.72)
Z_a [c]	4	1
Refinement statistics		
Resolution (Å)	50-2.7	50-2.9
Reflections (work)	28,423	32,610
Reflections (test)	2,117	2,360
R_{cryst}(%) [d]	22.8	19.3
R_{free}(%) [e]	27.3	24.1
Average B-value (Å^2)	39.0	90
Protein atoms	6498	7222
Carbohydrate atoms	0	0
Waters	0	0
RMSD from ideal geometry		
Bond length (Å)	0.011	0.012
Bond angles (°)	1.49	1.48
Ramachandran statistics (%) [g]		
Favored	93.1	90.43
Outliers	2.3	1.63
PDB ID	XXX [g]	XXX [g]

[a] *Numbers in parentheses refer to the highest resolution shell.*

[b] $R_{sym} = \Sigma_{hkl} \Sigma_i | I_{hkl,i} - \langle I_{hkl} \rangle | / \Sigma_{hkl} \Sigma_i I_{hkl,I}$ *and* $R_{pim} = \Sigma_{hkl} (1/(n-1))^{1/2} \Sigma_i | I_{hkl,i} - \langle I_{hkl} \rangle | / \Sigma_{hkl} \Sigma_i I_{hkl,I}$, *where* $I_{hkl,i}$ *is the scaled intensity of the* i^{th} *measurement of relection h, k, l,* $\langle I_{hkl} \rangle$ *is the average intensity for that reflection, and* n *is the redundancy (62).*

[c] Z_a *is the number of either Fab, HA monomer or HA monomer-Fab complexes per crystallographic asymmetric unit.*

[d] $R_{cryst} = \Sigma_{hkl} | F_o - F_c | / \Sigma_{hkl} | F_o | \times 100$

[e] R_{free} *was calculated as for* R_{cryst}, *but on a test set comprising 5% of the data excluded from refinement.*

[f] *Calculated using Molprobity (53)*

[g] *Coordinates and structure factors are deposited in the PDB and are available immediately on publication.*

Suppl. Table 4: Data collection and refinement statistics.

Acknowledgments

We are grateful to Drs. Richard Webby and Scott Kraus from the St. Jude Children's Research Hospital, Memphis, TN, USA, Yves Thomas and Laurent Kaiser from University Hospital of Geneva, Switzerland, and Rodney Daniels from the National Institute of Medical Research, London, UK, for kindly providing viruses form their repository.

References

1. Tong, S.*, et al.*, *A distinct lineage of influenza A virus from bats.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(11): p.4269-74.
2. Okada, J.*, et al.*, *Localization of epitopes recognized by monoclonal antibodies that neutralized the H3N2 influenza viruses in man.* J Gen Virol, 2011. **92**(Pt 2): p. 326-35.
3. Xu, R.*, et al.*, *Structure, receptor binding, and antigenicity of influenza virus hemagglutinins from the 1957 H2N2 pandemic.* J Virol, 2010. **84**(4): p. 1715-21.
4. Caton, A. J.*, et al.*, *The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype).* Cell, 1982. **31**(2 Pt 1): p. 417-27.
5. Tsuchiya, E.*, et al.*, *Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus.* J Gen Virol, 2001. **82**(Pt 10): p. 2475-84.
6. Wang, T. T.*, et al.*, *Seroevidence for H5N1 Influenza Infections in Humans: Meta-Analysis.* Science, 2012. **335**(6075):p.1463.
7. Tweed, S. A.*, et al.*, *Human illness from avian influenza H7N3, British Columbia.* Emerg Infect Dis, 2004. **10**(12): p. 2196-9.
8. Butt, K. M.*, et al.*, *Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003.* J Clin Microbiol, 2005. **43**(11): p. 5760-7.
9. Subbarao, K.*, et al.*, *Scientific barriers to developing vaccines against avian influenza viruses.* Nat Rev Immunol, 2007. **7**(4): p. 267-278.
10. Zhu, H.*, et al.*, *Infectivity, Transmission, and Pathology of Human H7N9 Influenza in Ferrets and Pigs.* Science, 2013. **341**(6142):p. 183-6.
11. Okuno, Y.*, et al.*, *A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains.* J Virol, 1993. **67**(5): p. 2552-8.
12. Dreyfus, C.*, et al.*, *Structure of a classical broadly neutralizing stem antibody in complex with a pandemic H2 hemagglutinin.* J Virol, 2013. **87**(12):p. 7149-54.
13. Sui, J.*, et al.*, *Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses.* Nat Struct Mol Biol, 2009. **16**(3): p. 265-73.
14. Ekiert, D. C.*, et al.*, *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(5924): p. 246-51.
15. Ekiert, D. C.*, et al.*, *A Highly Conserved Neutralizing Epitope on Group 2 Influenza A Viruses.* Science, 2011. **333**(6044):p. 843-50.
16. Corti, D.*, et al.*, *A Neutralizing Antibody Selected from Plasma Cells That Binds to Group 1 and Group 2 Influenza A Hemagglutinins.* Science, 2011. **333**(6044):p. 850-6.
17. De Marco, D.*, et al.*, *A Non-VH1-69 Heterosubtypic Neutralizing Human Monoclonal Antibody Protects Mice against H1N1 and H5N1 Viruses.* PLoS ONE, 2012. **7**(3): p. e34415.
18. Throsby, M.*, et al.*, *Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells.* PLoS One, 2008. **3**(12): p. e3942.
19. Dreyfus, C.*, et al.*, *Highly Conserved Protective Epitopes on Influenza B Viruses.* Science, 2012. **337**(6100):p. 1343-8.

20. Hu, W., *et al.*, *Fully human broadly neutralizing monoclonal antibodies against influenza A viruses generated from the memory B cells of a 2009 pandemic H1N1 influenza vaccine recipient.* Virology, 2013. **435**(2): p. 320-8.
21. Lingwood, D., *et al.*, *Structural and genetic basis for development of broadly neutralizing influenza antibodies.* Nature, 2012. **489**(7417): p. 566-70.
22. Fleishman, S. J., *et al.*, *Computational design of proteins targeting the conserved stem region of influenza hemagglutinin.* Science, 2011. **332**(6031): p. 816-21.
23. Whitehead, T. A., *et al.*, *Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing.* Nat Biotechnol, 2012. **30**(6): p. 543-8.
24. McLean, G. R., *et al.*, *Recognition of human cytomegalovirus by human primary immunoglobulins identifies an innate foundation to an adaptive immune response.* Journal of immunology, 2005. **174**(8): p. 4768-78.
25. Faber, C., *et al.*, *Three-dimensional structure of a human Fab with high affinity for tetanus toxoid.* Immunotechnology : an international journal of immunological engineering, 1998. **3**(4): p. 253-70.
26. Newton, K., *et al.*, *Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies.* Cell, 2008. **134**(4): p. 668-78.
27. Al-Lazikani, B., *et al.*, *Standard conformations for the canonical structures of immunoglobulins.* J Mol Biol, 1997. **273**(4): p. 927-48.
28. Thomson, C. A., *et al.*, *Germline V-genes sculpt the binding site of a family of antibodies neutralizing human cytomegalovirus.* The EMBO journal, 2008. **27**(19): p. 2592-602.
29. McLean, G. R., *et al.*, *Autoreactivity of primary human immunoglobulins ancestral to hypermutated human antibodies that neutralize HCMV.* Molecular immunology, 2006. **43**(12): p. 2012-22.
30. Torán, J. L., *et al.*, *Molecular analysis of HIV-1 gp120 antibody response using isotype IgM and IgG phage display libraries from a long-term non-progressor HIV-1-infected individual.* Eur J Immunol, 1999. **29**(9): p. 2666-75.
31. Hoffmann, E., *et al.*, *Universal primer set for the full-length amplification of all influenza A viruses.* Arch Virol, 2001. **146**(12): p. 2275-89.
32. Hoffmann, E., *et al.*, *A DNA transfection system for generation of influenza A virus from eight plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6108-13.
33. Barbas, C. F., 3rd, *et al.*, *Phage Display: A Laboratory Manual*. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
34. Stevens, J., *et al.*, *Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus.* Science, 2004. **303**(5665): p. 1866-70.
35. Smith, K., *et al.*, *Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen.* Nat Protoc, 2009. **4**(3): p. 372-84.
36. McCoy, A. J., *Solving structures of protein complexes by molecular replacement with Phaser.* Acta Crystallogr D Biol Crystallogr, 2007. **63**(Pt 1): p. 32-41.
37. Murshudov, G. N., *et al.*, *Refinement of macromolecular structures by the maximum-likelihood method.* Acta Crystallogr D Biol Crystallogr, 1997. **53**(Pt 3): p. 240-55.
38. Emsley, P., *et al.*, *Features and development of Coot.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 4): p. 486-501.
39. McDonald, I. K., *et al.*, *Satisfying hydrogen bonding potential in proteins.* J Mol Biol, 1994. **238**(5): p. 777-93.
40. Sheriff, S., *et al.*, *Structure of myohemerythrin in the azidomet state at 1.7/1.3 A resolution.* J Mol Biol, 1987. **197**(2): p. 273-96.
41. Connolly, M. L., *Solvent-accessible surfaces of proteins and nucleic acids.* Science, 1983. **221**(4612): p. 709-13.
42. Abhinandan, K. R., *et al.*, *Analysis and improvements to Kabat and structurally correct numbering of antibody variable domains.* Mol Immunol, 2008. **45**(14): p. 3832-9.

43. Chen, V. B., *et al.*, *MolProbity: all-atom structure validation for macromolecular crystallography.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 1): p. 12-21.

4.2 A novel pan flu monoclonal antibody in functional comparison with other broadly cross-reactive, neutralizing mAbs recognizing a conserved epitope on Influenza A hemagglutinin

This is a manuscript in preparation. I contributed in the following way: I prepared the phage display library by cloning the genetic material from isolated B cells; mutated, cloned, expressed, purified and biotinylated the H7 hemagglutinin used for panning; performed selection; expressed and purified mAb 1.12 and some of the hemagglutinins used for subsequent assays; purified most of the virus stocks; validated mAb 1.12 in binding, neutralization, epitope mapping and kinetic assays; performed in vivo protection assays. I also prepared figures and wrote every part of the manuscript.

A novel pan flu monoclonal antibody in functional comparison with other broadly cross-reactive, neutralizing mAbs recognizing a conserved epitope on Influenza A hemagglutinin

Wyrzucki A[1], Blattmann B[3], Steck M[1], Bianchi M[1], Kohler I[1], Grütter M[3], Hangartner L[1].

[1]Institute of Medical Virology, University of Zurich, Switzerland; [3]Department of Biochemistry, University of Zurich, Switzerland

Summary

Influenza A viruses can tolerate changes in their antigenic structures very well, and are capable of rapidly escaping pre-existing immunity. Here we describe a novel monoclonal antibody (clone 1.12) selected from a human phage-display library that, in contrast to the majority of antibodies elicited by vaccination or natural infection, has a broad neutralizing activity encompassing all 15 subtypes tested. Competition assays using murine c179 antibody revealed that 1.12 interacts with a similar, conserved epitope in the stem of hemagglutinin. Kinetic experiments showed that virus neutralization by mAb 1.12 and two other heterosubtypic antibodies is irreversible. However, potent neutralization is possible only if the antibodies bind to HA spikes before virus attaches to the cell surface. Furthermore, we did not find interference between HA-reactive human sera and heterosubtypic antibodies, indicating that pre-existing immunity would not hamper the access of these antibodies to the membrane-proximal region of hemagglutinin. Accordingly, these data suggest that heterosubtypic antibodies are likely to be effective in influenza exposed individuals once elicited by a properly designed immunogen.

Introduction

Hemagglutinin (HA) and neuraminidase (NA), the two major surface antigens of Influenza A virus, rapidly change in a process known as antigenic drift [1]. This enables the virus to escape pre-existing immunity causing seasonal epidemics and local outbreaks. Besides acquiring mutations, the HA and NA genome segments can also be exchanged between subtypes, leading to the emergence of new reassortant viruses. Viruses arising from this process known as antigenic shift have caused several pandemics in the past with the 1918-1919 ‘Spanish flu’ outbreak being the most severe [2, 3]. As a preventive measure, influenza vaccines have been used for over 70 years now [1, 4, 5]. Although quite effective against seasonal influenza, currently available vaccines have several limitations. One major drawback is that antibodies elicited by the seasonal vaccine only react with the inoculated and

closely related strains. This strain-specific (or homotypic) nature of the antibody response implies that seasonal vaccines have to be often reformulated to reflect antigenic changes acquired by drifting. As an outcome, the vaccine manufacturing process is time consuming and the delivered vaccine may be ineffective if mismatch occurs. Therefore an extensive effort has been made in the last two decades to formulate an immunogen that would provide protection against multiple Influenza A subtypes. Such vaccine would be beneficial not only during seasonal outbreaks but also against pandemic strains that are insensitive to seasonal vaccination due to change of HA or NA subtype.

The development of universal Influenza A vaccine has been approached over the years using several different strategies [6, 7]. One of the most promising is the elicitation of high titers of broadly cross-reactive antibodies to hemagglutinin (HA) [8-11]. HA is the major surface antigen of Influenza A and is crucial for virus entry into the host cell. There are currently 17 different subtypes of HA that are divided into two phylogenetic groups. The high immunogenicity of the protein makes HA an interesting target for vaccine design. Multiple studies showed that antibodies elicited against HA are sufficient to provide protection from virus infection [12, 13]. However, as mentioned above, the cross-reactivity of induced sera is very limited. This arises from the fact that the majority of elicited antibodies bind the highly variable head of HA [14]. However, during last 20 years highly conserved epitopes could be identified on the Influenza A hemagglutinin, and their discovery is believed to facilitate the design of a universal Influenza A vaccine [15]. In the past 4 years several monoclonal antibodies cross-reactive to many HA subtypes have been described [16-22]. However, of these only two mAbs, clones FI6 and CR9114, were binding the majority of HA subtypes from both phylogenetic groups. Detailed analysis revealed that all heterotypic antibodies react with HA stem, thus providing a structural basis for the design of a universal Influenza A vaccine.

In this study we decided to broaden the knowledge about the binding properties of heterosubtypic antibodies. We identified a novel heterosubtypic mAb, termed 1.12, from the antibody repertoire of a healthy donor using a phage display library and a modified HA antigen. Remarkably, clone 1.12 neutralized multiple Influenza A virus strains belonging to HA subtypes 1 to 15. The viruses used in our neutralization assay were isolated over the last 80 years, which emphasizes the high conservation of the epitope recognized by mAb 1.12. As expected, binding competition assay revealed that 1.12 interacts with HA stem with an epitope overlapping that previously described for stem-reactive mAb c179 [17, 23]. Furthermore, mAb 1.12 protected mice from lethal challenge with A/Puerto Rico/8/1934 (H1N1) virus proving its *in vivo* efficacy. In addition, we showed that at least three heterosubtypic antibodies irreversibly neutralize the A/Puerto Rico/8/1934 (H1N1) virus, and their action is not restricted in the presence of pre-existing serum antibodies binding to HA. Therefore our data suggest that HA stem-reactive, heterotypic mAbs can easily access their epitopes on intact virus

particles also in a situation when HA spikes are densely covered with strain-specific homotypic antibodies. Thus we conclude that heterotypic antibodies elicited by a designed immunogen would be effective in protecting individuals that have already been exposed to Influenza A.

Results

Isolation of mAb 1.12

We have used a Fab phage display library based on the quiescent B cell repertoire of a healthy human subject. The library has been panned against an immobilized and modified HA protein using standard procedure (see materials and methods for details). A single phage display selection with 4 subsequent selection rounds was performed, and a total of 48 clones from the 3rd and 4th round were analyzed for ELISA binding to recombinantly expressed H2 (A/Japan/305/1957), H3 (A/Moscow/10/1999) and H7 (A/ Fowl plague /Bratislava/1979) hemagglutinins. Remarkably, 43 out of 48 clones showed cross-reactivity to all tested HAs. Sequence analysis revealed that all selected clones have almost invariant heavy chains (HC) derived from the VH 1-69 germline. Interestingly, all positive clones have been identified with heavy chain complementarity determining region 3 (HCDR3) that contains a long stretch of five adjacent tyrosine residues. In contrast, a high variability and the use of different germlines have been seen for light chains (LC). Thus we assumed that most possibly the heavy chain only is responsible for binding to HA, and randomly selected one clone termed 1.12.

Binding and neutralizing activity of mAb 1.12

To further analyze the cross-reactivity of clone 1.12, it was expressed as a soluble IgG_1 molecule in 293T cells. In ELISA, binding of a purified IgG1 to a panel of recombinant hemagglutinins from both phylogenetic groups was observed (Figure 1). mAb 1.12 bound all HAs (H1, H2, H3, H4, H5, H7, H12) with EC_{50} values ranging from 126 ng/ml to 979 ng/ml. The strongest binding was seen for the H1, the weakest for H12 hemagglutinin. Next, the neutralizing activity of mAb 1.12 was determined. A total of 19 Influenza A isolates from 15 HA subtypes was assessed. We deliberately chose life virus for these assays as pseudotyped viruses have been shown to be easier to neutralize [14]. We have found that mAb 1.12 neutralized all tested viruses at IC_{50} values ranging from 107 ng/ml to 23471 ng/ml. No neutralization of an influenza B isolate was observed. Thus, mAb 1.12 neutralized all Influenza A viruses tested and its epitope is obviously shared between at least 15 out of 17 HA subtypes

and has not been subject to antigenic drift since at least 1934, when the oldest strain tested was isolated.

Epitope recognized by mAb 1.12

Based on the structural data published for the previously described heterotypic antibodies binding HA we suspected that also mAb 1.12 is interacting with the HA stem [16-21, 23]. Indeed, in an ELISA binding competition assay using the prototypic stem-reactive mAb c179, c179 was outcompeted by mAb 1.12 in a concentration-dependent manner (Figure 2). Since no obvious structural changes in HA were found in the crystal upon binding of mAb C179 to hemagglutinin, it is safe to assume that the epitope of both antibodies overlap. Detailed structural and mutagenesis data are necessary to provide a solid proof for our findings.

When the impact of glycosylation at position 291 and a proline at position 293 was assessed, it was found that the neutralizing activity of mAb 1.12 against a reassortant PR8 virus carrying the HA gene segment from A/duck/Memphis/546/74 (H11N9) in which the glycosylation site at position 291 has been removed, it was about 10-fold more sensitive to neutralization by mAb1.12 than the glycosylated wt HA protein (IC_{50} 17.6 µg/ml vs. 2.4µg/ml).

In vivo activity and pharmacokinetics of mAb 1.12

We have evaluated the protective efficacy of mAb 1.12 in C57BL/6 mice in a prophylactic setup (Figure 3). To this end mice were injected IP with 10 or 3 mg/kg of mAb 1.12 24h before intra nasal infection with a 2xLD_{50} dose of A/Puerto Rico/8/1934 (H1N1) virus. At 10 mg/kg 8 out of 10 animals were protected whereas at 3 mg/kg only 1 out of 10 mice succumbed to infection. Furthermore, surviving mice showed no apparent signs of morbidity and only displayed a moderate weight loss. Interestingly, the time point of antibody application had an influence on protection by mAb 1.12. Intra peritoneal application 3h before infection conferred the lowest level of protection whereas intra venous injection 2h before infection was protective to all animals at both tested doses (15 and 5 mg/kg, 3 mice per group). To further understand the aforementioned differences mice were injected intravenously with 5 or 15 mg/kg of mAb 1.12. Serum antibody levels were determined at different time points post injection. Based on the collected data we conclude that serum half-life of mAb 1.12 is very satisfactory and the reported differences in level of protection can be most possibly attributed to slow diffusion of mAb 1.12 in tissues.

Functional comparison of mAbs 1.12, 3.1, FI6

To gain insight into the functional and kinetic properties of HA-stem reactive antibodies, several experiments were performed using a test panel of heterosubtypic mAbs: 1.12, 3.1, and FI6. First, to assess the reversibility of neutralization we preincubated A/Puerto Rico/8/1934 (H1N1) virus in solution with a fixed concentration of mAbs from our set (Figure 4). Next we captured the virus-mAb particles on magnetic beads and performed 3 different dissociation protocols: 'no dissociation' where captured virus is incubated in the presence of the antibody for 17 h, 'short dissociation' where antibodies were washed away just before adding the captured virus to cells and 'long term dissociation' where antibodies were washed away 14 h before the virus was added to cells. As depicted in Figure 4, even after prolonged dissociation time, virus neutralization was complete and thus indistinguishable from virus that was permanently incubated in the presence of the antibodies. Therefore we concluded that the heterosubtypic epitope is accessible on free virus particles in solution, and that neutralization of the infectivity is not reversible, either due to irreversible damage introduced to HA or due to little or no dissociation from the complex.

In the 2nd assay, we tested whether heterosubtypic antibodies still have access to their epitope when virus particles are already bound to the cell surface (Figure 5). In two parallel setups we evaluated the neutralizing activity of mAb 1.12, 3.1 and FI6 on the A/Puerto Rico/8/1934 (H1N1) as free virus in solution and virus already pre-adsorbed on cell surface. Surprisingly, neutralization of pre-adsorbed virus on cell surface was much less efficient than that of free virions. This was seen for all tested mAbs suggesting that HA stem-reactive antibodies cannot effectively access their epitopes on hemagglutinin spikes once the virus is attached to cells.

Since it has been speculated that apically binding strain-specific antibodies may sterically restrict access to the membrane-proximal heterosubtypic epitopes, we tested if antibodies from our panel can efficiently neutralize virus particles that have been saturated with human serum antibodies (Figure 6). To this end, A/Puerto Rico/8/1934 (H1N1) virus was incubated with human sera at concentrations that were subneutralizing but were confirmed to provide saturating binding to H1 PR8 HA coated on ELISA plates. As a control, virus was mock incubated without serum. These virus preparations were then incubated with our panel of heterosubtypic mAbs at two limiting dilutions (10 and 1 µg/ml). We have found that no difference between the applied setups can be seen. Thus, these data demonstrate that human serum antibodies do not interfere with neutralization of Influenza A virus by HA stem-reactive heterosubtypic mAbs. Furthermore, we could see an additive neutralizing effect when both serum and our mAbs have been applied at subneutralizing concentrations.

Discussion

In this study we isolated a new pan-Influenza A antibody mAb 1.12 and evaluated its antiviral properties in comparison with two other heterotypic mAbs binding to the hemagglutinin stem. Broadly cross-reactive mAb 1.12 was isolated from a combinatorial phage display library that has been prepared from quiescent B-cells isolated from an average human individual. Since previous isolations of such antibodies typically involved brute-force approaches, we were reasonably surprised how easy it was to select multiple Fab clones with a very broad cross-reactivity to hemagglutinins from both phylogenetic groups of Influenza A in a single panning experiment [21]. This ease can be attributed to several factors. First, we used a modified hemagglutinin that was designed such that access of antibodies to the highly variable head was sterically impaired, while the conserved epitopes in the stem were prominently exposed. Second, we noticed that the antigen, whose design was based on the HA from phylogenetic group 2, selected heterotypic antibodies of broad specificity while the antigen based on an HA protein from phylogenetic group1 only isolated antibodies that are specific for strains from phylogenetic group 1. This indicates that the strain on which an antigen-design is based may have a great impact on the antibodies selected. However, we are currently conducting further studies to dissect the impact of either factor on the antibodies isolated in more detail. Moreover, we are currently testing these antigens as immunogens in mice.

mAb 1.12 showed remarkable cross-neutralizing activity to 15 out of 17 subtypes of Influenza A virus. Furthermore, clone 1.12 neutralized multiple isolates from each of the H1, H2, H3, H6 and H7 subtypes. As the panel of viruses tested in our study represents almost 80 years of antigenic drift of Influenza A we conclude that the epitope recognized by antibody 1.12 is almost invariant across this genus. However, no apparent neutralization was seen for A/Shorebird/Delaware/172/06(H16N3) that also grew only very poorly in our hands. Since H16 is circulating in wild birds and up till now it was not identified in humans, we do not believe that this shortcoming is relevant for the potential use of this antibody as therapeutic agent. Moreover, similarly impressive breadth like that of clone 1.12 has been previously only reported for Influenza A and B-specific antibody CR9114 [18]. Interestingly, these antibodies share several features. First, they both recognize epitopes localized in HA stem. Second, they primarily utilize their heavy chains to bind hemagglutinin. Third, both have a stretch of at least four tyrosine residues in their HCDR3, that in case of CR9114 interact with the N-terminal residues of HA2.

Using 1.12 together with two other heterosubtypic mAbs, we have addressed several questions that are central for an estimation of the potential success of any vaccination eliciting heterosubtypic antibodies. It has been speculated that the tight arrangement of the HA and NA spikes

on the virion surface may make access to the membrane proximal heterosubtypic epitopes difficult. Moreover, this access may even be further restricted if the virions are saturated with homotypic antibodies, binding to the strain-specific apical epitopes, elicited during previous infections or vaccinations. However, we did not find that homotypic, serum antibodies interacting with hemagglutinin head interfere with the neutralizing activity of heterosubtypic antibodies. Accordingly, we do not predict that a universal influenza vaccine or therapy based on stem-reactive, heterotypic antibodies would be impaired by the presence of pre-existing strain-specific humoral immunity.

When we assessed the reversibility of the neutralization by our set of heterosubtypic mAbs, we have found that at least H1N1 viruses are neutralized irreversibly. At this point we can only speculate about the reason for this irreversibility. One possibility would be that the stem reactive antibodies have a very slow dissociation rate thus, once bound to HA, they stay attached to it over a long period of time. Alternatively, prolonged binding of these antibodies may induce conformational changes that render the HA protein non-functional. Such mechanism of neutralization has been seen for the aforementioned gp120-specific mAbs [24]. Further investigation would be necessary to describe the neutralization mechanism in details.

Quite unexpectedly, we did find that heterosubtypic antibodies cannot efficiently neutralize virus particles that are already attached to the cell surface. We speculate that based on the postulated aggregation of estimated six HA molecules required for fusion (three of which have to undergo a conformational change; [25]) a cluster of HA molecules is formed to whose center there is no access for heterosubtypic antibodies. Accordingly, a sufficient number of HA spikes still can undergo the confirmation change to mediate infection.

The discovery of a novel pan Influenza A antibody altogether with a detailed evaluation of binding mechanism of heterosubtypic mAbs provides further insight into the subject of conserved epitopes on hemagglutinin. The data collected in our study may be essential for the design of a new immunogen and furthermore justify the use of HA as valuable antigen in the preparation of a universal influenza vaccine.

Materials and methods

Library construction and phage display selection of cross-reactive Fab clones

The phage library was prepared as described in [26]. In brief, frozen PBMCs from donor RI13 were used to purify B cells using anti-CD22 coated MACS beads (~1.6×10^6 B cells isolated). Following

total RNA extraction (RNeasy Mini, Qiagen), reverse transcription into cDNA was performed using oligo dT primer (Promega) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. Rearranged variable gene segment families were amplified individually and modified for phage surface expression in 3 subsequent PC reactions. Resulting full-length Fab fragments were cloned into the pComb3X phage display vector, and used to rescue a phage library with a total of $1.5x10^9$ transformants giving rise to $3.3x10^{11}$ phage particles/ml library titer.

This phage display library was enriched for phages binding to the antigenically altered antigens immobilized on streptavidin coated magnetic beads (construction and biochemical characterization of these antigens will be reported elsewhere; beads were purchased form Promega). Approximately $2.5x10^{12}$ of phages were combined with bead-immobilized hemagglutinin (see below, f.c. of protein= 100 nM as counted for HA dimer) in the first round of selection. In total 4 rounds of selection were performed with 100 nM HA concentration (as counted for HA trimer in rounds 2-4) and increasing wash stringency (wash with TBST 0.05% Tween 20). Phage clones obtained from 3rd and 4th round were screened for binding to various HAs in ELISA experiment. Positive clones were sequenced.

Expression and purification of recombinant HAs

Recombinant HA was expressed in SF9 cells using baculovirus vectors as described by Stevens or Ekiert [27]. In brief, HA open reading frames were modified to contain an insect secretion sequence at the N-termial, and foldon trimerization instead of a transmembrane and intracellular domain at the C-terminal end. Secreted recombinant protein was purified from cell supernatant 4 days post infection on NiNTA columns (GE healthcare). To process recombinant HA protein into HA1 and HA2, it was trypsin digested using 10U of TPCK-treated trypsin (from bovine pancrease, Sigma Aldrich) at 1 µg of HA, at RT for 1h. Immediately after trypsinization, protein was purified by size exclusion chromatography on a S200 gel filtration column (GE healthcare). For further experiments only the fraction corresponding to HA trimer was used. All proteins used in phage display had Cys residue introduced at position 158 in the head region to enable biotinylation (with EZ-link HPDP biotin, Pierce) and coupling to streptavidin beads in upside-down orientation. The proteins were further processed as described in phage display panning section.

Expression and purification of IgG1

For expression of soluble IgG_1 1.12, 3.1 and FI6, the sequence of HC and LC were cloned into pAbvec Ig plasmids [28, 29]. Proteins were expressed by transfecting 293T cells (30 µg of each HC and LC plasmids per 1 T150 flask) using PEI as transfection reagent. Cell supernatants were harvested,

sterile filtered at 0.2 µm before recombinant IgG_1 was purified via affinity chromatography using protein G slurry (GE healthcare). The column eluted IgGs (with 100 mM glycine, pH 2,7) were rebuffered to PBS by dialysis using Spectra/Por 1 membrane (Spectrumlab) or three rounds of buffer exchange on centrifugal filter (Amicon Ultra-15).

ELISA

Binding of IgG 1.12 to various HAs was assessed by ELISA. To this end, high binding, half-area plates (Costar) were coated with 25 µl/well of 2-4 µg/ml HA in PBS at 4 °C overnight. Plates were then blocked with 60 µl of 2% low-fat dry milk in PBS for 1h at RT. Purified IgG was titrated in 0.2% milk PBS, and incubated in a volume of 30 µl on the coated plates at RT for 1h. Bound IgG was detected using goat anti-human kappa-HRP secondary antibody (1:5000, Southern Biotech). Between each step, plates were washed 4 times with approx. 200 µl of TBST (0.1% Tween). ELISA signal was developed using ultra TMB substrate (Pierce) for 5-10 min before the reaction was stopped by the addition of 2N H_2SO_4. OD450 was measured in the Perkin Elmer plate reader. As negative control, recombinantly expressed HIV gp120-specific IgG_1 b12 was included in all assays.

Competition ELISA

ELISA plates (half area, high binding, Costar) were coated with 25 µl/well of 2 µg/ml of H1 PR8 HA in PBS at 4 °C ON. Plates were then blocked with 60 µl of 2% milk in PBS at RT for 1h. Blocked plates were incubated with 30 µl of serially diluted (in PBS/0,2% milk) human IgG 1.12 at RT for 1h and washed 3x with TBST. Further, the plates were incubated at RT for 1h with the murine IgG c179 diluted in PBS/0,2% milk at 1 µg/ml and washed 4x with TBST. Binding of IgG 1.12 and IgG c179 was detected in parallel using goat anti-human kappa-HRP polyclonal serum (Southern Biotech) or polyclonal rabbit anti-mouse-HRP serum (Dako), respectively. Signal was developed as described in the ELISA section.

Neutralization of Influenza A viruses

Titrated IgG 1.12 was mixed with a fixed amount of Influenza A virus corresponding to MOI 2-3 (~100000 pfu/well) in DMEM medium supplemented with 0,2% BSA, 20 mM HEPES, P/S, Glu (D/B/H medium). After 2h at 37°C in cell culture incubator mAb-virus mixture was transferred on PBS washed, subconfluent MDCK cells growing on 96-well tissue culture plates (TPP). To enable infection cells were kept in 37°C CO_2 incubator for 1h. Next, the mix was removed from cells that were later washed with PBS and supplemented with D/B/H medium. After 5-7h (depending on the growth kinet-

ics of virus isolate) in 37°C CO_2 incubator cells were fixed with methanol, washed and stained with HB65-FITC (mAb detecting Influenza A NP) diluted in 1% BSA PBS at 3 µg/ml, 4°C, ON. FITC fluorescence signal was detected using Perkin Elmer plate reader. The b12 gp120 HIV- specific antibody was used as negative control.

Reversibility of neutralization

Highly concentrated, sucrose-cushion purified stock of A/Puerto Rico/8/1934 (H1N1) virus was diluted 1:30 in 900 µl of D/B/H medium and mixed with 100 µl of mAb dilution in the same medium in a way that the final mAb concentration was 10 µg/mL. The mixture was incubated in CO_2 incubator at 37°C for 2h. In the meantime 120 µL of 1:10 diluted magnetic beads coated with α-N1 neuraminidase antibody was blocked with 120 µL of 2% milk in PBS at RT on rotator for 1h. After both incubation steps milk solution was removed, beads were washed with PBS, combined with the virus-mAb mixture and kept in CO_2 incubator at 37°C on rotator for 1h. Next, 300 µL of the 1mL beads-virus-mAb mixture was transferred into 3 tubes. From now on each tube represents one of the test conditions: 'no dissociation', 'short-term dissociation' and 'long-term dissociation'. Long-term dissociation beads were washed 1 x with 400 µL of D/B/H medium and resuspended in 1 ml of D/B/H medium without antibody. At the same time 'no dissociation' and 'short-term dissociation' tubes were filled up to 1 ml with D/B/H medium supplemented with appropriate mAb (final concentration 10 µg/ml). Next, tubes were kept in CO_2 incubator at 37°C on rotator for 14 h. After incubation the supernatant was removed and 100 µL of D/B/H medium supplemented with 10 µg/mL of mAb added to 'no dissociation' tube. In parallel the 'short term dissociation' and 'long term dissociation' samples were washed with D/B/H medium and resuspended in 100 µl of D/B/H medium. 50 µL of resuspended beads was next transferred on MDCK cells seeded at density 1-2e4/well in 96 well plate a day before. Plates with cells were kept in CO_2 incubator at 37°C for 30 min, placed on orbital shaker for few sec to resuspend the beads and transferred back to incubator for another 30 min. After the incubation beads were again resuspended on orbital shaker, withdrawn and cells washed 1x with PBS. Further incubation and detection of infectivity was performed as described for neutralization assay. The HIV gp120-specific antibody b12 was used as non-neutralizing control.

Neutralization of virus particles attached to cell surface

MDCK cells were seeded with a number of 1-2x10^4/well a day before the experiment. At the day of experiment plates were transferred to fridge (4°C) for 15 min and later on ice for 15 min to ensure that no virus internalization will occur in the subsequent step. Next, cells were washed with

ice-cold PBS and infected at MOI~3 with A/Puerto Rico/8/1934 (H1N1) virus at 4°C for 1,5 h ('virus preadsorbed on cells' plate). Another MDCK plate was prepared in parallel using the same procedure but no virus was added ('virus in solution' control sample). Both plates were next washed 1x with ice cold PBS. mAbs titrated in D/B/H medium were further added to 'virus preadsorbed on cells' plate. Ice cold D/B/H medium was added to the 'virus in solution' control plate. Next, both plates were incubated at 4°C for 2h. In parallel A/Puerto Rico/8/1934 (H1N1) virus (from the same initial dilution kept at 4°C) at MOI~3 was mixed with cold titrated mAbs on dilution plate and incubated at 4°C for 2h. Further, both MDCK-containing plates were 1x washed with prewarmed PBS. 'Virus preadsorbed on cells' plate was covered with 100 µL of prewarmed D/B/H medium whereas the virus+mAb mixture from titration plate was transferred to the 'virus in solution' control plate. Infectivity was later detected as described in the neutralization assay. The HIV gp120-specific antibody b12 was used as control.

Competition with human sera

20 µl of 1:405 diluted in D/B/H medium, concentrated, sucrose-cushion purified A/Puerto Rico/8/1934 (H1N1) virus was first combined with 20 µl of appropriately diluted human serum and incubated in CO_2 incubator at 37°C for 1h (final serum dilutions are indicated in table below). At this step 2 dilutions for each serum were used- the 'optimal' dilution which shows low neutralization and the 'neutralizing' dilution which provides suboptimal virus neutralization. Each dilution was confirmed beforehand to have a saturated binding to H1 PR8 recombinantly expressed HA in ELISA experiment. After incubation 20 µl of appropriate mAb was added to virus-serum mix to a final concentration of 10 or 1 µg/ml and the mixture was kept in CO_2 incubator at 37°C for 1h. Next, 50 µl of the suspension was transferred on MDCK cells and incubated in CO_2 atmosphere at 37°C for 1h to enable infection. Infectivity was later detected as described in the neutralization assay.

Serum no	Optimal dilution (1st)	Neutralizing dilution (2nd)
58	1:270	1:90
75	1:100	1:33
511	1:270	1:90
579	1:270	1:90

Figures

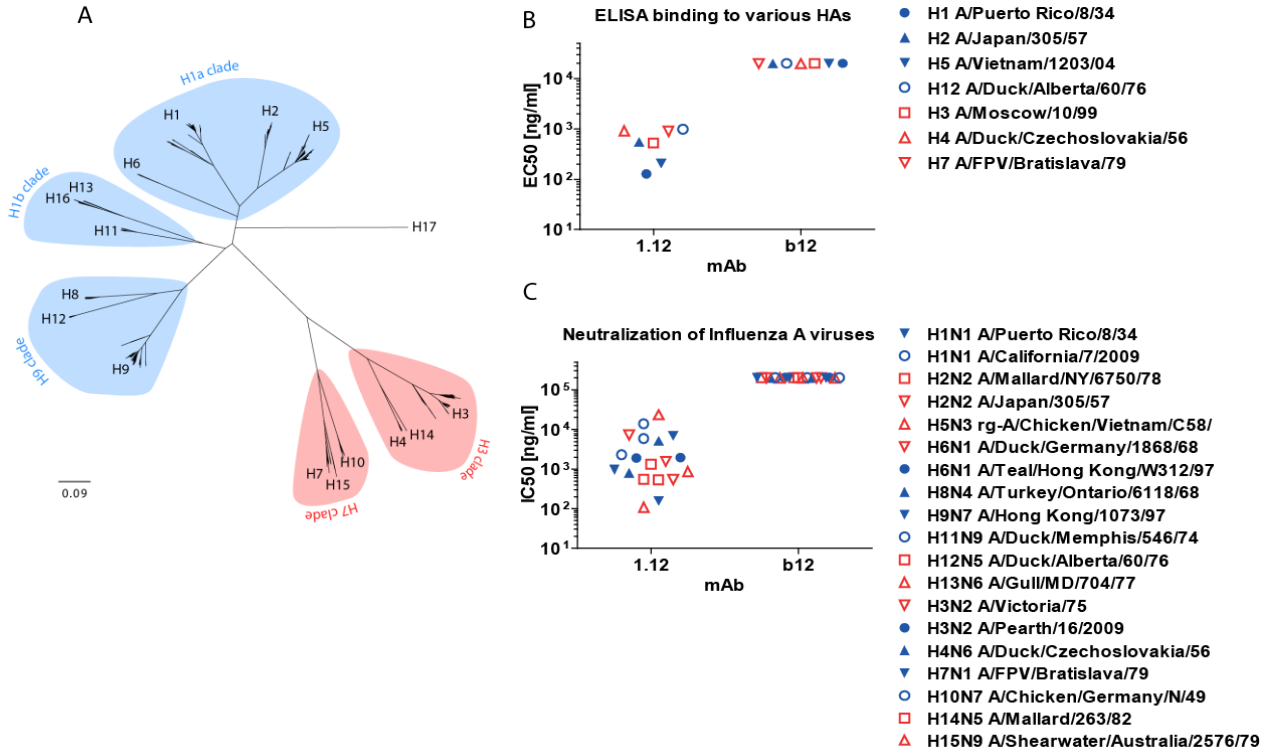


Figure 1. Binding and neutralizing activity of mAb 1.12. A) Phylogeny of Influenza A hemagglutinin. Two phylogenetic groups can be distinguished that encompass multiple clades that in turn split into individual subtypes. Phylogenetic group 1 is indicated in blue, group 2 in red. B) half-maximum binding (EC50) of mAb 1.12 to 7 distinct HA subtypes was measured by ELISA. C) A panel of 20 viruses covering 16 subtypes of Influenza A has been tested for neutralization by mAb 1.12. The antibody concentration that neutralized 50% of the viral inoculums (IC50) is indicated. HIV-1 gp120-specific mAb b12 was used as a negative control in both experiments. Values above 10^4(A) or 10^5(B) indicate no binding or neutralization, respectively. For subtype H16 no neutralization by mAb1.12 was seen.

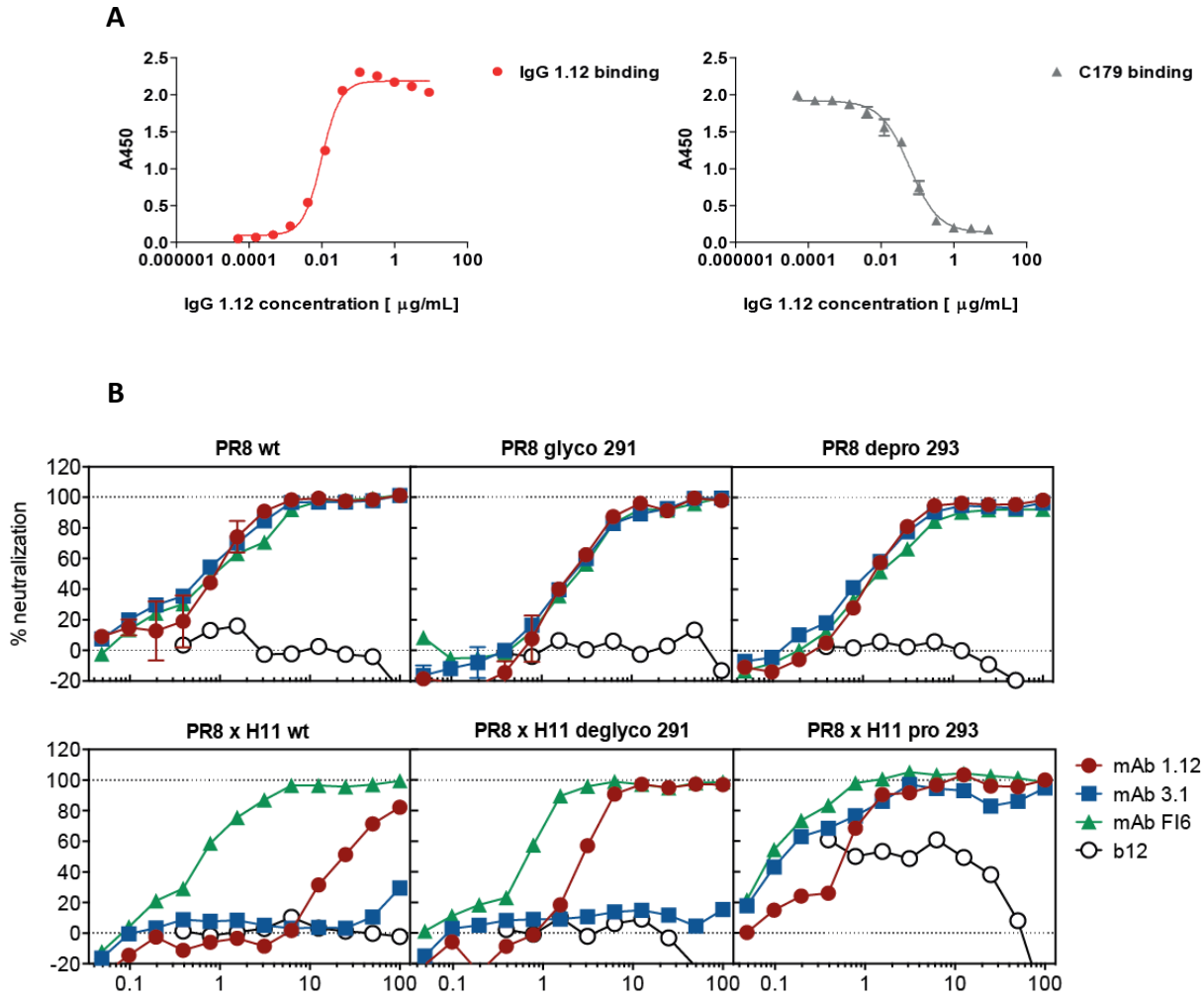


Figure 2. Epitope mapping. A) The epitope recognized by mAb 1.12 was roughly evaluated in a binding competition ELISA assay using HA stem-reactive mAb C179. ELISA plates coated with purified HA from A/Puerto Rico/8/34(H1N1) was incubated with titrated amounts of mAb 1.12, washed and later incubated with a fixed concentration (1 µg/ml) of the murine mAb C179. Binding of both antibodies was next detected using different secondary polyclonal sera. B) The impact of glycosylation and presence of Pro residue in HA stem on activity of mAbs 1.12, 3.1 and FI6 was measured in a infectivity reduction assay using various Influenza A mutants and reassortant viruses.

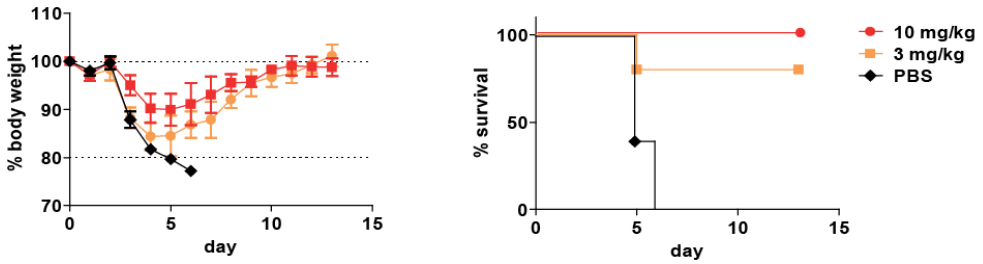


Figure 3. Prophylactic protection of mice by mAb 1.12 A) Mice were injected intraperitoneally with the indicated dose of mAb 1.12 in PBS or with PBS alone (control group) 24h prior to intranasal infection with a lethal dose of A/Puerto Rico/8/1934(H1N1) virus ($2x10^3$ TCID50 units). Mice dropping below 80% of initial body weight were scored as dead and euthanized. Each group contained 5 BL6 females, a representative of at least 2 consistent experiments is shown.

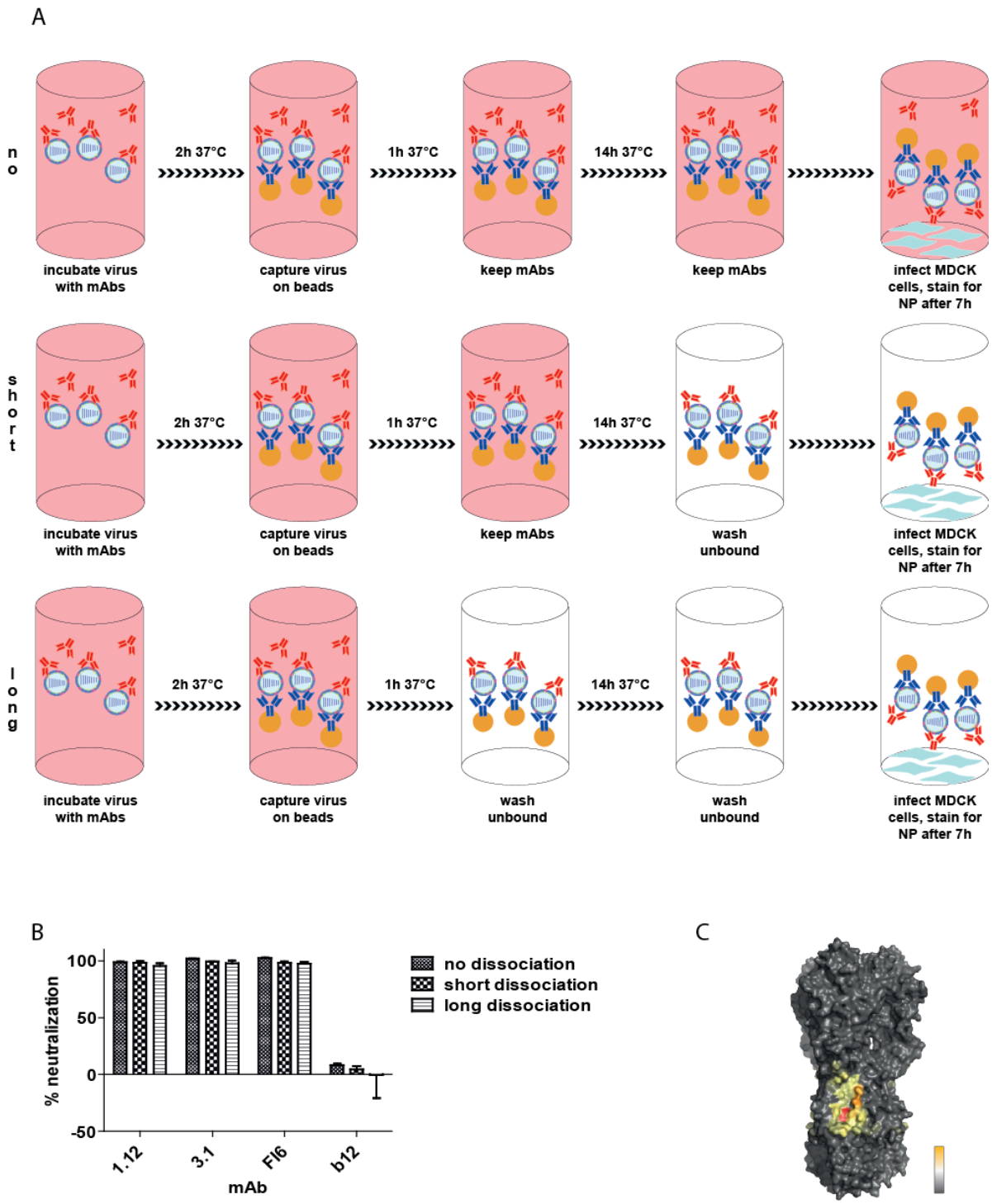
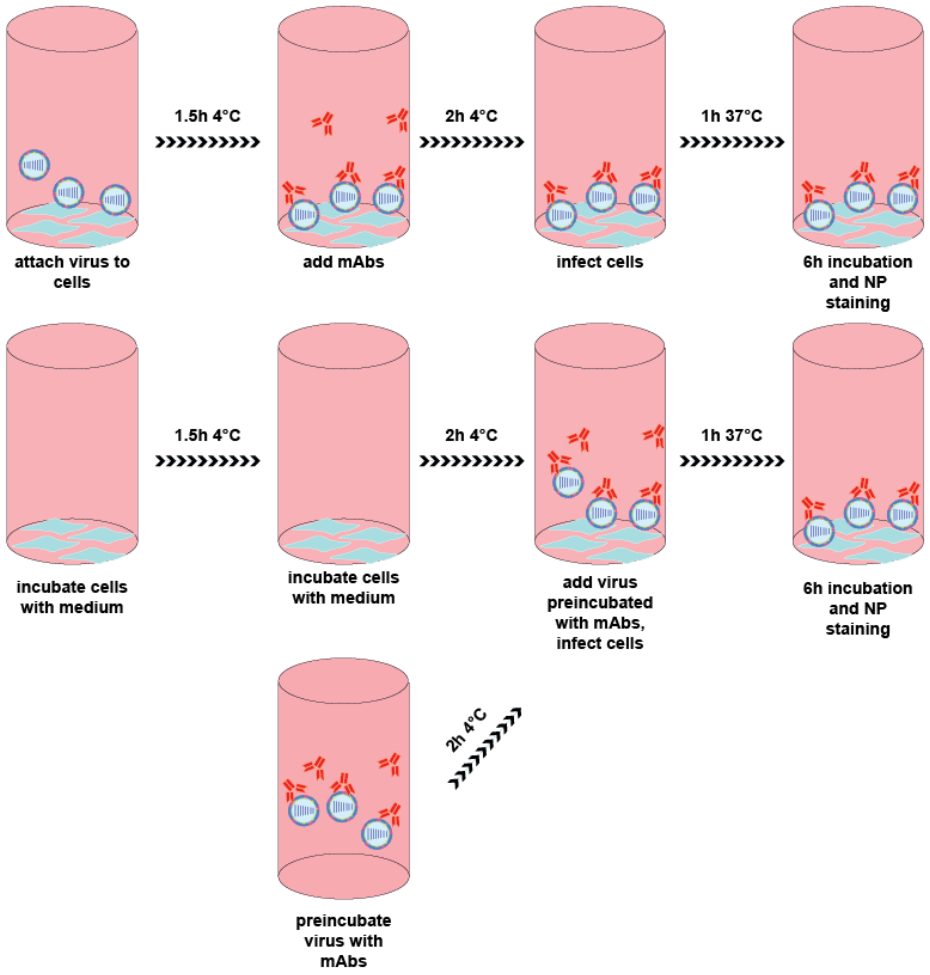


Figure 4. Reversibility of Influenza A neutralization. Reversibility of neutralization has been tested as depicted in diagram (A). Three HA stem-reactive antibodies were incubated at concentration of 10 µg/ml with A/Puerto Rico/8/1934(H1N1) virus (amount corresponding to MOI ~ 30) and the mAb-virus mixture was captured on magnetic beads. Beads were then processed using different procedures mimicking 'no dissociation', 'short dissociation' and 'long dissociation' conditions. In the last step infectivity of each sample was measured in neutralization assay with MDCK cells. The HIV-1 gp120-specific mAb b12 was used as negative control (B). (C) Surface representation of the conserved epitope on HA stem. Colors from gray (not involved) to orange (frequently contacted) indicate the surface recognized by stem-reactive, broadly neutralizing antibodies. Red indicates the location of Trp21.

A


1.5h 4°C
2h 4°C
1h 37°C
attach virus to cells
add mAbs
infect cells
6h incubation and NP staining
1.5h 4°C
2h 4°C
1h 37°C
incubate cells with medium
incubate cells with medium
add virus preincubated with mAbs, infect cells
6h incubation and NP staining
2h 4°C
preincubate virus with mAbs

B

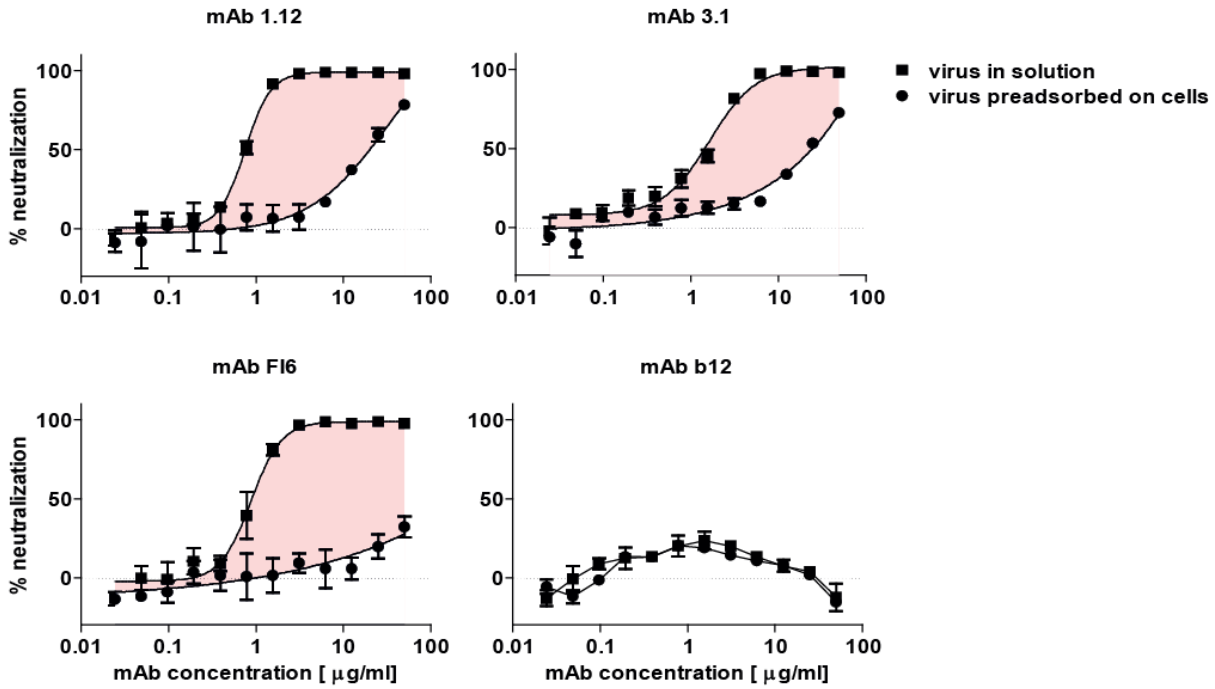

mAb 1.12
mAb 3.1
virus in solution
virus preadsorbed on cells
% neutralization
mAb FI6
mAb b12
mAb concentration [µg/ml]
mAb concentration [µg/ml]

Figure 5. Neutralization of virus attached to cell surface. Diagram (A) represents the experimental setup used to test neutralization of virus particles attached to cell surface. The A/Puerto Rico/8/1934 (H1N1) virus has been pre-adsorbed on MDCK cells at 4^{o}C to avoid virus internalization. Attached viruses were then incubated with titrated amounts of HA stem reactive mAbs and residual infectivity was detected. As a control, viruses were mock-incubated in cell-free medium before titrated amounts of the mAbs were added. The HIV-1 gp120-specific mAb b12 was used as non-neutralizing control.

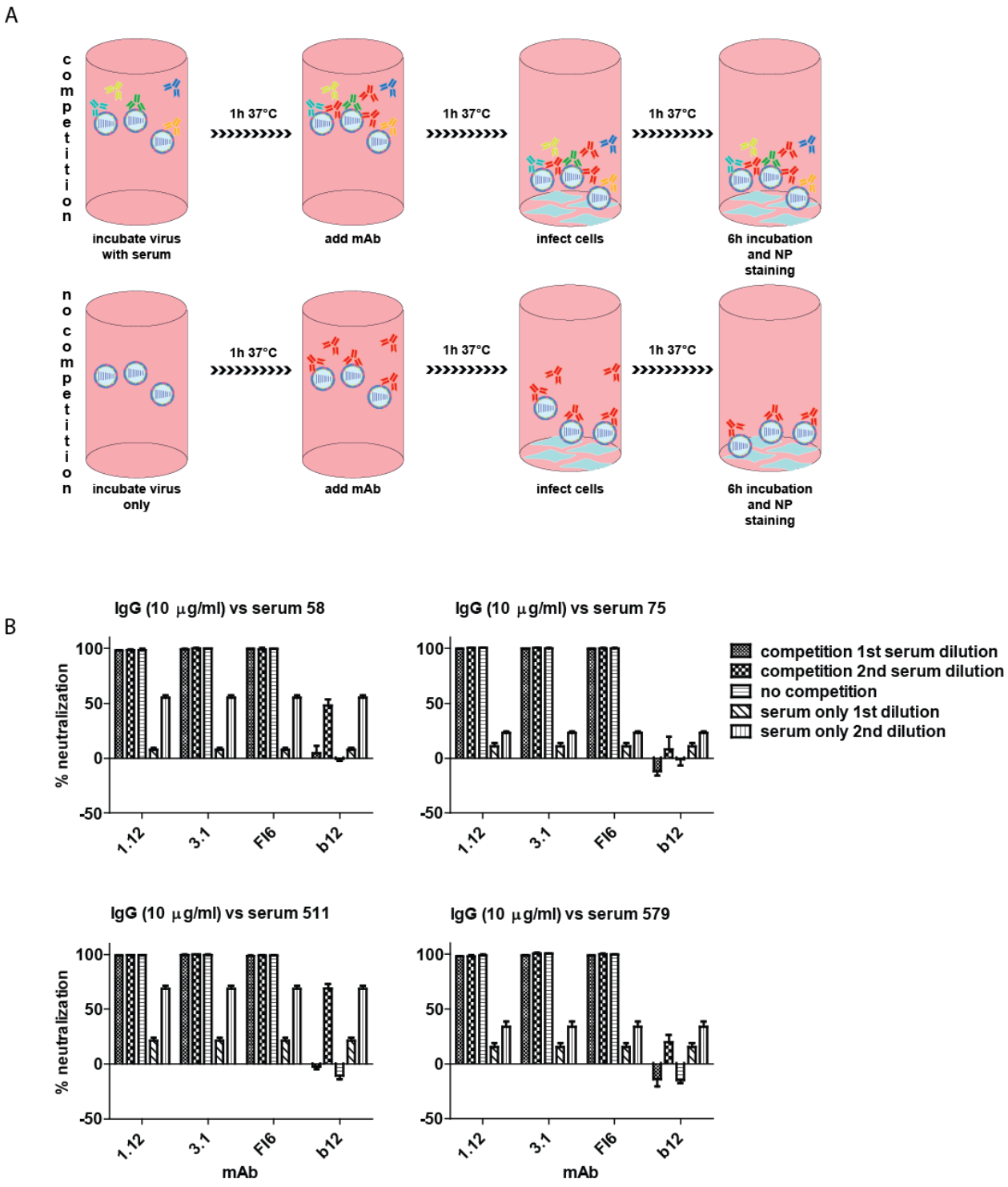


Figure 6. Competition with human sera. Competition for neutralization between human sera and HA stem-reactive mAbs was performed as depicted in diagram (A). A/Puerto Rico/8/1934 (H1N1) virus has been first incubated with serum from the indicated donor for 1 h before the indicated mAb was added at a concentration of 10 µg/ml. Residual infectivity of the sample was evaluated and compared to infectivity of sample processed the same way but without the addition of human serum. Two serum dilutions were chosen: 1st dilution corresponds to a serum concentration giving saturated signals in ELISA while only having minor neutralizing activity on the virus. The 2nd ('neutralizing') serum concentration is three times higher than the 1st.

Acknowledgements

We are grateful to Drs. Richard Webby and Scott Kraus from the St. Jude Children's Research Hospital, Memphis, TN, USA, Yves Thomas and Laurent Kaiser from University Hospital of Geneva, Switzerland, and Rodney Daniels from the National Institute of Medical Research, London, UK, for kindly providing viruses form their repository. We would also like to thank Prof. Alexandra Trkola and her group for support and providing reagents.

References

1. Fields, B. N.*, et al.*, *Fields virology, 5th edition*, 2007.
2. Nguyen-Van-Tam, J. S.*, et al.*, *The epidemiology and clinical impact of pandemic influenza.* Vaccine, 2003. **21**(16): p. 1762-8.
3. Monto, A. S., *Epidemiology of influenza.* Vaccine, 2008. **26 Suppl 4**: p. D45-8.
4. Nichol, K. L., *Efficacy and effectiveness of influenza vaccination.* Vaccine, 2008. **26 Suppl 4**: p. D17-22.
5. Osterholm, M. T.*, et al.*, *Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis.* Lancet Infect Dis, 2012. **12**(1): p. 36-44.
6. Palese, P., *Making better influenza virus vaccines?* Emerg Infect Dis, 2006. **12**(1): p. 61-5.
7. Pica, N.*, et al.*, *Toward a universal influenza virus vaccine: prospects and challenges.* Annu Rev Med, 2013. **64**: p. 189-202.
8. Krammer, F.*, et al.*, *Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly-protective stalk-specific antibodies.* J Virol, 2013. **87**(12):p. 6542-50.
9. Steel, J.*, et al.*, *Influenza virus vaccine based on the conserved hemagglutinin stalk domain.* MBio, 2013. **1**(1).
10. Wang, T. T.*, et al.*, *Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18979-84.
11. Bommakanti, G.*, et al.*, *Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge.* Proc Natl Acad Sci U S A, 2010. **107**(31): p. 13701-6.
12. Clements, M. L.*, et al.*, *Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus.* J Clin Microbiol, 1986. **24**(1): p. 157-60.
13. Scherle, P. A.*, et al.*, *Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells.* J Immunol, 1992. **148**(1): p. 212-7.
14. Corti, D.*, et al.*, *Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine.* J Clin Invest, 2010. **120**(5): p. 1663-73.
15. Ekiert, D. C.*, et al.*, *Broadly neutralizing antibodies against influenza virus and prospects for universal therapies.* Curr Opin Virol, 2012. **2**(2): p. 134-41.
16. Sui, J.*, et al.*, *Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses.* Nat Struct Mol Biol, 2009. **16**(3): p. 265-73.
17. Dreyfus, C.*, et al.*, *Structure of a classical broadly neutralizing stem antibody in complex with a pandemic h2 influenza virus hemagglutinin.* J Virol, 2013 **87**(12): p. 7149-54.
18. Dreyfus, C.*, et al.*, *Highly conserved protective epitopes on influenza B viruses.* Science. **337**(6100): p. 1343-8.
19. Ekiert, D. C.*, et al.*, *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(5924): p. 246-51.

20. Ekiert, D. C*., et al.*, *A Highly Conserved Neutralizing Epitope on Group 2 Influenza A Viruses.* Science, 2011. **333**(6044):p. 843-50.
21. Corti, D*., et al.*, *A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins.* Science, 2011. **333**(6044): p. 850-6.
22. Hu, W*., et al.*, *Fully human broadly neutralizing monoclonal antibodies against influenza A viruses generated from the memory B cells of a 2009 pandemic H1N1 influenza vaccine recipient.* Virology, 2013. **435**(2): p. 320-8.
23. Okuno, Y*., et al.*, *A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains.* J Virol, 1993. **67**(5): p. 2552-8.
24. Ruprecht, C. R*., et al.*, *MPER-specific antibodies induce gp120 shedding and irreversibly neutralize HIV-1.* J Exp Med, 2011. **208**(3): p. 439-54.
25. Dobay, M. P*., et al.*, *How many trimers? Modeling influenza virus fusion yields a minimum aggregate size of six trimers, three of which are fusogenic.* Mol Biosyst, 2011. **7**(10): p. 2741-9.
26. Barbas, F. C. III, *et al.*, *Phage display: a laboratory manual.* 2001.
27. Ekiert, D. C*., et al.*, *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(5924): p. 246-51.
28. Wardemann, H*., et al.*, *Predominant autoantibody production by early human B cell precursors.* Science, 2003. **301**(5638): p. 1374-7.
29. Wrammert, J*., et al.*, *Rapid cloning of high-affinity human monoclonal antibodies against influenza virus.* Nature, 2008. **453**(7195): p. 667-71.

4.3 Apendix – Immunizations with modified Influenza A hemagglutinin proteins induce broadly cross-reactive sera, and provide protection against heterologous virus challenge in mice

This is a short report from the currently ongoing study. I contributed to this project in the following way. I designed immunogens used for vaccination, initially tested the efficacy of those immunogens in mouse model, developed and tested a large scale system for hemagglutinin production, performed the experiment showing that there is no interference between the vaccine-elicited sera and the stem reactive mAb 1.12. A series of follow-up studies including different immunogen designs and various vaccination procedures as well as mouse challenge experiments with heterologous virus was performed by Dr. Matteo Bianchi.

Encouraged by the findings from the phage display selection experiments we decided to use the same antigen design to vaccinate mice to elicit antibodies that are cross-reactive with different hemagglutinin subtypes, and that may provide protection against a variety of Influenza A isolates. To this end, C57BL/6 mice were immunized twice using 35 or 55 µg of the same modified group 1- or group 2-derived hemagglutinins, as were used for phage display (i.e. inverted, coupled to magnetic beads). We then tested the serum for binding to the same two hemagglutinins in ELISA and found that the sera cross-react with both HAs with higher antibody titers seen for the group-2 derived antigen (Figure 1). However, sera from vaccinated mice were not neutralizing the A/Puerto Rico/8/1934 (H1N1) virus *in vitro.* As it was later tested, the mouse sera did not compete for the binding to hemagglutinin with the stem-reactive, heterosubtypic mAb 1.12 suggesting low binding affinities of the serum antibodies, or the presence of alternative heterosubtypic but non-neutralizing epitopes. In a follow up study performed by Dr. Bianchi it has been shown that sera from vaccinated mice are cross-reactive to a broad panel of HA from both phylogenetic groups (H1, H2, H3, H4, H5, H7, H12) and that antibody titers can be further increased by introducing adjuvants to the vaccine formulation. Furthermore, it has been proven that the group 1- and 2-derived antigens performed comparably in a non-inverted immobilization whereas omitting the coupling to magnetic beads had a strong negative impact on antigen performance. Moreover, although only weak neutralization to a homologous virus strain could be seen with the mouse sera, mice were protected from a lethal challenge with a heterologous viral isolate. The study is currently continued focusing on proving the heterotypic protection provided by our antigens.

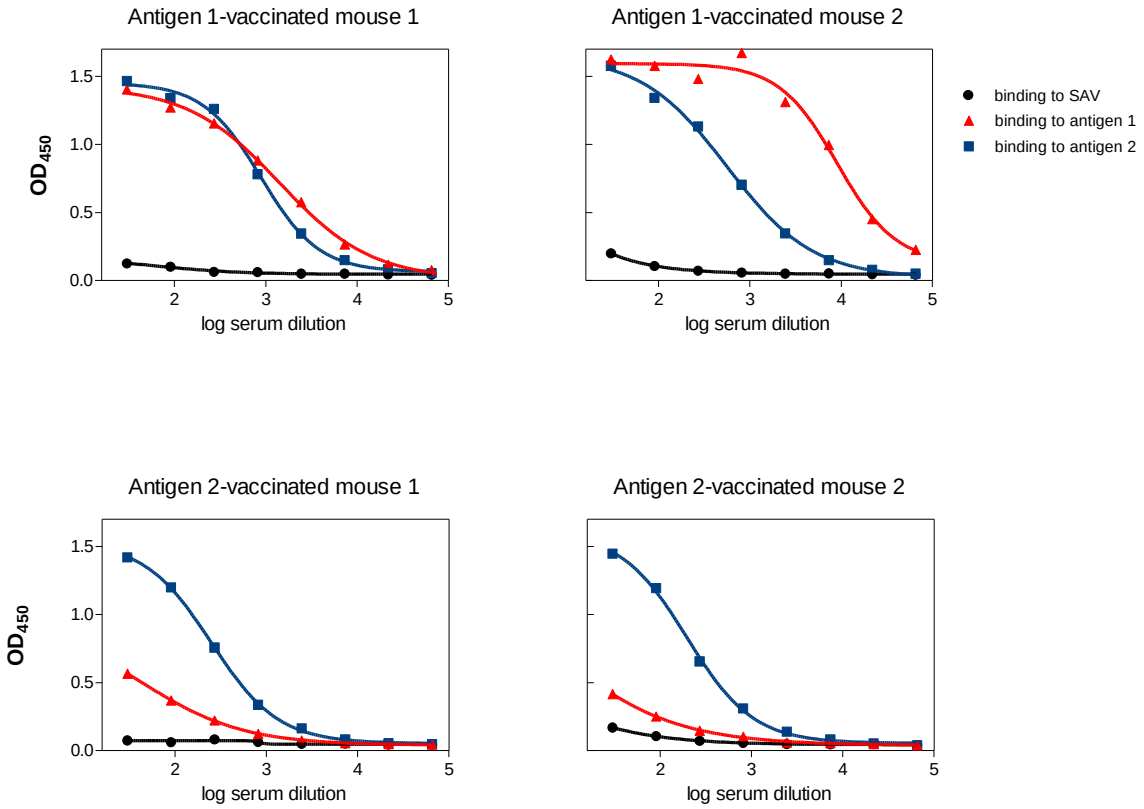


Figure 1. Cross-reactivity of mice sera after two IP injections with modified hemagglutinins. The cross-reactivity has been evaluated in ELISA using recombinant hemagglutinins and strepatavidin (SAV) as indicated on graph.

5. Discussion

5.1 Discovery of two new heterosubtypic Influenza A antibodies

Due to its considerable impact on human health, economics and agriculture, Influenza A viruses have been widely studied since their discovery [24, 25, 34]. The major focus of these studies was the development of prophylactic and therapeutic agents that could restrict the global spread of seasonal and pandemic viruses. Although much progress was achieved, the highly variable nature of Influenza A makes it a constant threat, and requires the development of novel treatment and prevention strategies. In the past 2 decades, a significant effort has been made to engineer drugs and vaccines that are effective against a broad range of viruses belonging to multiple subtypes [82, 84, 93, 94]. The greatest challenge in this approach, however, is the selection of a proper viral component that could be exploited as target for prevention and therapy. The primary requirement for such component is that it represents a highly conserved structure that can be readily accessed by newly designed drugs, or that it possesses a high immunogenicity, in case of novel vaccine formulations. Both, immunogenicity and accessibility of hemagglutinin (HA), the major surface antigen of Influenza A virus, make it a very suitable research target. However, for a long time it has not been known whether HA contains conserved epitopes that are shared between all subtypes and that would be useful for the design of novel immunogens. In 1993, a murine antibody, clone c179, has been described to neutralize viruses belonging to several HA subtypes from the phylogenetic group 1, and this antibody provided the first indication that the epitope recognized by mAb c179 is a part of HA stem and is actually conserved [68]. In the last four years, several human heterotypic antibodies binding to HA stem have been reported and the existence of hemagglutinin epitopes with a high level of conservation, accessibility and immunogenicity confirmed [48, 51, 58, 75, 78, 79, 129]. Noteworthy, two of these mAbs, FI6 and CR9114, showed cross-reactivity to multiple virus subtypes from both phylogenetic groups, protected from lethal challenge *in vivo* and also displayed *in vitro* neutralization.

At the start of the present study, only little was known about heterosubtypic epitopes, and it was the goal to broaden this knowledge. Moreover, it was planned to apply my findings in the design of a universal Influenza A vaccine candidate. Thus, I developed novel antigens and confirmed the integrity of their conserved epitopes by isolation and characterization of naturally occurring antibodies that can bind to these antigens. To this end, I have used phage display to enrich and isolate antibodies that originally were elicited by natural infection or vaccination. The design of these antigens took several considerations into account: First, they were based on recombinant full-length hemag-

glutinin proteins since previous attempts using truncated 'headless' HA proteins have failed [117, 121]. Second, to avoid enrichment of normal strain-specific antibodies, the design of the antigens was based on non-human or extinct HA subtypes, implying that all antibodies binding to these antigens are *bona fide* heterosubtypic [119]. Last, the antigens were designed in a way that access to the highly immunogenic and variable HA head was sterically blocked and that the conserved stem was prominently exposed.

The phage display enrichment experiment indicated that my design considerations, and their implementation indeed worked out very well. One selection event was sufficient to capture two sets of clones presenting two different binding specificities. Out of these I have chosen one representative for each set (clone 1.12 and 3.1) and further characterized them in multiple assays. To my surprise, mAb 1.12 neutralized multiple isolates belonging to 15 out of 17 existing HA subtypes, confirming the highly conserved nature of the epitope recognized by this antibody. Moreover, the panel of neutralized viruses was isolated over the last 80 years, further supporting the highly conserved nature of the epitope recognized by clone 1.12. Of note, mAb 1.12 was not effective in neutralization of A/Shorebird/Delaware/172/2006 (H16N3). This can be partially explained by the fact that the H16 subtype circulates only in wild birds and therefore phage display library based on human B cell repertoire would not contain an antibody capable of recognizing H16 hemagglutinin. In contrast, the second isolated clone, mAb 3.1, potently neutralized only subtypes from phylogenetic group 1 (H1, H2, H5, H6). As expected, several epitopes mapping experiments, including solving the crystal structure for mAb 3.1 in complex with H1 from A/South Carolina/1918 (H1N1), revealed that both antibodies bind to the HA stem. Moreover, IgG_1 1.12 and 3.1 provided protection to mice challenged with a lethal dose of a heterologous H1N1 virus. Thus, these data show that my antibodies are very similar to the recently described heterosubtypic mAbs. Taken together, all these findings indicate the presence of highly conserved, protective, immunogenic structures in the hemagglutinin stem that could be utilized for the design of a universal drug or vaccine against a broad range of Influenza A viruses.

Interestingly, amongst heterosubtypic, HA-reactive antibodies isolated to date, heavy chains encoded by either the VH 1-69 or VH 3-30 germline family are overrepresented [48, 51, 58, 75, 78, 79, 129]. The heavy chains of mAbs 1.12, F10, CR6261 are representatives of VH 1-69 germline encoded antibodies which have also been reported to encode for some broadly cross-reactive HIV gp120 mAbs. An interesting feature of the V_H 1-69 is the presence of conserved, hydrophobic residues that are crucial for engaging with HA and gp120 at the tip of HCDR2 [72]. Thus, based on several studies the V_H 1-69 germline has been proposed to have evolved towards recognition of hydrophobic patches present on various viral antigens. In fact, it has been shown that Ig molecules containing the V_H 1-69 germline sequence, when expressed as B cell receptor on the surface of B cells, initiate the B

cell receptor signal cascade following binding of hemagglutinins. Further, only a low number of somatic mutations in the V_H 1-69 gene are sufficient to generate heterotypic antibodies potently neutralizing Influenza A viruses. In contrast, the extend to which a V_H 3-30 gene has to be hypermutated to be able to potently block different virus subtypes depends on antibody. For instance, clone 3.1 carries a low number of hypermutations in its HC and it utilizes almost exclusively its heavy chain for binding. In contrast, mAb FI6 is heavily hypermutated in its heavy chain and also uses its light chain for binding to hemagglutinin [75]. Based on the limited set of data collected for FI6 and 3.1 it may be speculated that the neutralizing breadth of V_H 3-30 antibodies is proportional to the amount of introduced hypermutations.

5.2 Design of universal Influenza A vaccine

The ultimate goal of this study was to design an immunogen that could provide protection against challenge with different virus subtypes, and that could later be developed into a universal Influenza A vaccine. As outlined above, the probably most promising approach is to use modified hemagglutinin constructs for immunization. Work in this field has been especially boosted in the last 4 years due to the discovery of numerous broadly cross-reactive antibodies and the molecular characterization of their highly conserved epitopes on HA. Various artificial immunogens that were supposed to display such epitopes have been developed [93, 117, 119-121, 130]. Unfortunately, to date only moderate success has been reported. These immunogens only triggered limited cross-reactivity and failed to induce neutralizing antibodies. Poor *in vivo* protection was the major problem when using recombinant hemagglutinins in vaccine formulations. Thus, as outlined above, for my study I decided to pursue a different approach for the design of hemagglutinin-based immunogens. I took advantage of the same set of two antigens that performed remarkably well in phage display selection yielding heterotypic antibodies. Due to their covalent up-side-down tethering to beads, access to the variable head is blocked, and the conserved stem is prominently exposed. Moreover, due this formulation of the antigen, also a rigid and repetitive antigen pattern that has been shown to favor B cells responses is provided [131, 132]. Indeed, when mice were vaccinated with either immunogen, they developed high titers of serum antibodies that were cross-reactive to all hemagglutinin subtypes tested so far (H1, H2, H3, H4, H5, H7, H12). Although the elicited sera exhibited week neutralization only towards the homologous vaccine strain, immunized mice were protected from lethal challenge with a heterologous H1N1 virus. Similar *in vivo* protection by non-neutralizing antibodies has been previously reported in several studies, and it is assumed that it is mediated by neutralization-independent antibody effector functions such as complement activation and antibody-dependent cellular cytotoxicity [75, 110, 111]. From competition ELISA experiments we know that the mouse

serum antibodies do not compete with mAb 1.12. This suggests that the majority of induced antibodies bind outside the epitope recognized by clone 1.12. I therefore postulate the existence of one or several other, conserved, non-neutralizing epitopes on the surface of hemagglutinin. Alternatively, the remarkable breadth of the elicited antibodies comes at the price of a poor affinity/off-rate that is not able to compete with the monoclonal antibody for binding. Additional animal studies are being currently performed to evaluate the potency of our immunogen against challenge with other viral strains. Moreover, detailed epitope mapping is being applied to better understand which surface areas of our immunogens are inducing antibodies present in sera of vaccinated mice.

Although both immunogens used in our study performed well in capturing or eliciting cross-reactive antibodies, I can not state which of the design features is responsible for their high efficacy. The HA subtype seems to play a role, as in phage display selection the breadth of antibodies selected with the phylogenetic group 2-based antigen was greater than the one based on phylogenetic group 1. On the other hand both HA constructs performed comparably well in terms of eliciting high titers of cross-reactive sera in animal study. Similarly, utilizing the 'inverted' group 2-based construct with blocked access to HA head in phage display gave better results than in case of a 'non-inverted' version of the protein whereas no significant differences were seen for both immunogens in animal study. However, much higher titers of antibodies were induced by the construct when the protein was coupled to magnetic beads compared to immunization with soluble protein. This would indicate that either the beads themselves support the antibody production or the better humoral response is an effect of accumulating multiple HA molecules in an organized fashion on readily accessible surface. Since mixing beads with uncoupled antigen did not improve the immune response in preliminary experiments, it can be concluded that the coupling is indeed a considerable factor for the good immunogenicity of our immunogen.

5.3 Comparison of heterosubtypic antibodies binding to Influenza A hemagglutinin

5.3.1 Recognition of the conserved hemagglutinin epitope

To date, all heterotypic Influenza A antibodies, besides clone CR8020, were reported to recognize almost the same epitope on the stem of hemagglutinin [48, 51, 58, 71, 75, 78, 79]. This epitope consists of a hydrophobic grove that is formed by residues 18-52 and 290-330 in HA1 combined with aa 1-21 and 38-60 in HA2, and that shows a high degree of conservation within and between subtypes. Nevertheless, slight structural differences between clades and phylogenetic groups are present in this portion of HA, and these result in a various cross-reactivity pattern of heterotypic mAbs.

The primary differences comprise an altered orientation of Trp21 in HA2 ($Trp21_2$), the presence or absence of glycan at position 38 in HA1 as well as different residues at positions 49_2 and 111_2. Some of the antibodies like FI6 and CR9114 can overcome these limitations by employing highly flexible binding loops and by being able to displace the sugar side chains upon binding [75, 79]. Other antibodies such as CR6261, F10, and 3.1 fail to do so, which probably limits their breadth.

Interestingly, most antibodies use two types of heavy chains derived either from the germline V_H 1-69 or V_H 3-30. Moreover, recognition of residues in HA hydrophobic groove may be mediated by both heavy (HC) and light chain (LC) or exclusively by heavy chain with no specific pattern seen here. For example the antibodies 3.1 and FI6 utilize their both chains (3.1 uses the LC to a much lesser extend) but still present a very distinct cross-reactivity. However, some clear differences are seen for these closely related clones that account for their distinct breadth. First, clone 3.1 mainly uses hydrophobic residues from the HCDR1 and HCDR3 loops to contact the hydrophobic groove whereas FI6 almost exclusively interacts with its very long HCDR3. It seems that such HCDR3 has a significantly higher flexibility that allows the Phe residue at its tip to adopt different orientations for the crucial interaction with $Trp21_2$. A similar increased flexibility has also been reported for HCDR2 of the pan Influenza A and B antibody CR9114. In contrast the HC of clone 3.1 contains shorter CDR loops and is only moderately mutated comparing to the heavily hypermutated HC of FI6 that contains an addition of large amount of N-nucleotides. Thus, based on data summarized from our and previous studies, I can state that extensive hypermuation in the heavy chain can generate binding loops that are capable of recognizing a broader range of HA subtypes

Interestingly, both germline genes do not require hypermutation to bind or engage HA molecules [72, 75]. Furthermore, these germline genes have also been found in cross-reactive mAbs recognizing other viral antigens such as HIV, HCMV or SARS. Therefore, we believe that these two particular Ig germline families evolved to bind conserved elements on multiple viral glycoproteins at poor affinities, and that their specificity can then be further shaped by hypermutation into more affine antibodies with antiviral activity. Thereby, introduction of mainly hydrophobic residues in a proper context of HCDR binding loops may be crucial to recognize different viral antigens.

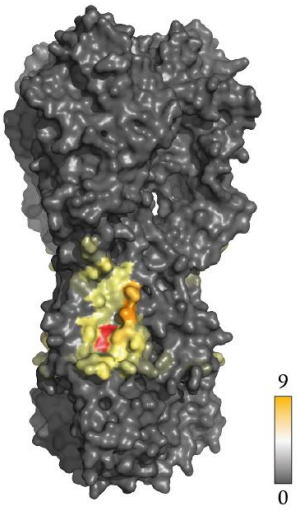


Figure 13. The conserved hemagglutinin epitope. Surface representation of the conserved epitope on HA stem. Colors from gray (not involved) to orange (frequently contacted) indicate the surface recognized by stem-reactive, broadly neutralizing antibodies. Red indicates the location of Trp21. Adapted from Lars Hangartner. Figure was prepared using PyMOL software (DaLano Scientific LLC).

5.3.2 Kinetics of neutralization

A wide range of heterosubtypic, neutralizing antibodies (nAbs) recognizing Influenza A hemagglutinin has been described in detail. Up till now, research in this field was mainly focused on the molecular characterization of the recognized epitope, and to some extent also on unraveling the mechanism by which HA-reactive nAbs abolish viral infectivity (either by preventing the HA-driven membrane fusion or by blocking the HA receptor binding site) [67, 129]. However, neutralization kinetics of these nAbs still remains elusive. I therefore decided to use my new heterosubtypic antibodies in combination with the pan Influenza A mAb FI6 to evaluate their mode of action in detail. To this end, I developed several different experimental *in vitro* setups that to some extend mimic crucial steps during natural infection. I reasoned that this type of data is essential to understand whether heterosubtypic antibodies binding to a membrane-proximal epitope would be effective for treatment and prevention of influenza A infections in humans, in particular if they are elicited by a future universal influenza vaccine. In the first of my experiments we asked if virus neutralization with HA stem-reactive antibodies is reversible. Such studies were previously performed for broadly reactive HIV-1 gp120-specific mAbs showing that some of them irreversibly neutralize viral infectivity [133]. Surprisingly, in my assay all antibodies from the test panel provided complete and irreversible neutralization of an H1N1 virus. At this point I do not know the exact mechanism behind this irreversibility. It is likely that those antibodies bind HA molecules with very low overall dissociation rates and thus keep the virus particles non-infective over a prolonged period of time. This would be in agreement with the measured high binding affinities for these antibodies. Fab fragments of these mAbs bind various hemagglutinins with K_D values in nanomolar range and even higher avidities can be expected for the corresponding IgG molecule. Taking into account the high density of HA spikes on the virus' surface

in combination with the high interaction affinities, I suspect that it would be difficult for HA-stem reactive antibodies to completely dissociate from the virus particle once they are bound to hemagglutinin. However, as revealed in my second experiment, complete neutralization can only be achieved if the mAbs bind to virus particles in solution. Once bound to the cell surface, I found that neutralization of viral infectivity required much higher antibody concentrations. I assume that HA spikes are occluded in the virological synapse (formed after the virus attaches to cellular membrane receptors) and are therefore more difficult to access for the mAbs. Nevertheless, based on the successful animal protection study I conclude that this limited neutralization of bound virus does not have a signifficant impact on protection, presumably because in mice, this shortcoming is compensated by the non-neutralizing effector functions of the transferred antibodies.

Since most humans display various amounts of homotypic antibodies induced by infection and vaccination, and since these antibodies are binding to apical epitopes, it was speculated that their binding would impair access of heterosubtypic mAbs to their membrane-proximal epitopes. Such interference by homotypic antibodies would have a great impact on the success of any therapeutic or preventive use of heterosubtypic antibodies. However, I could show that the presence of strain-specific human serum antibodies does not impact the neutralization activity of mAbs 1.12, 3.1 and FI6. Thus, my data suggest that from the mechanistic point of view, antibodies recognizing conserved epitopes on hemagglutinin stem would be effective as treatment option and when induced by a universal Influenza A vaccine.

The discovery of a novel heterosubtypic Influenza A antibodies altogether with a detailed evaluation of binding mechanism of these mAbs provides further insight into the subject of conserved epitopes present on hemagglutinin. The data collected may be essential for the design of a new immunogen. Moreover, these data justify the use of hemagglutinin as antigen capable of inducing heterosubtypic humoral response. Based on the knowledge and experience collected in our study we have prepared a set of vaccine candidates that performed surprisingly well in animal experiments. We are further evaluating and optimizing these designed immunogens aiming at development of a universal influenza vaccine.

6. References

1. Fields, B. N.*, et al.*, *Fields virology, 5th edition*, 2007.
2. CDC. *Influenza Type A Viruses and Subtypes*. 2013; Available from: http://www.cdc.gov/flu/avianflu/influenza-a-virus-subtypes.htm.
3. Palese, P., *Influenza: old and new threats.* Nat Med, 2004. 10(12 Suppl): p. S82-7.
4. Skehel, J. J.*, et al.*, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. 69: p. 531-69.
5. Bouvier, N. M.*, et al.*, *The biology of influenza viruses.* Vaccine, 2008. 26 Suppl 4: p. D49-53.
6. van de Sandt, C. E.*, et al.*, *Evasion of influenza A viruses from innate and adaptive immune responses.* Viruses, 2012. 4(9): p. 1438-76.
7. Claas, E. C.*, et al.*, *Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus.* Lancet, 1998. 351(9101): p. 472-7.
8. Fouchier, R. A.*, et al.*, *Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome.* Proc Natl Acad Sci U S A, 2004. 101(5): p. 1356-61.
9. Zebedee, S. L.*, et al.*, *Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions.* J Virol, 1988. 62(8): p. 2762-72.
10. Engelhardt, O. G.*, et al.*, *Functional association between viral and cellular transcription during influenza virus infection.* Rev Med Virol, 2006. 16(5): p. 329-45.
11. Yamaguchi, M.*, et al.*, *Zernike phase contrast electron microscopy of ice-embedded influenza A virus.* J Struct Biol, 2008. 162(2): p. 271-6.
12. Chen, W.*, et al.*, *A novel influenza A virus mitochondrial protein that induces cell death.* Nat Med, 2001. 7(12): p. 1306-12.
13. Lamb, R. A.*, et al.*, *Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins.* Proc Natl Acad Sci U S A, 1981. 78(7): p. 4170-4.
14. Baudin, F.*, et al.*, *Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent.* EMBO J, 1994. 13(13): p. 3158-65.
15. Rogers, G. N.*, et al.*, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin.* Virology, 1983. 127(2): p. 361-73.

16. Matrosovich, M. N.*, et al.*, *Human and avian influenza viruses target different cell types in cultures of human airway epithelium.* Proc Natl Acad Sci U S A, 2004. 101(13): p. 4620-4.
17. Gambotto, A.*, et al.*, *Human infection with highly pathogenic H5N1 influenza virus.* Lancet, 2008. 371(9622): p. 1464-75.
18. Martin, K.*, et al.*, *Transport of incoming influenza virus nucleocapsids into the nucleus.* J Virol, 1991. 65(1): p. 232-44.
19. Cros, J. F.*, et al.*, *An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein.* Traffic, 2005. 6(3): p. 205-13.
20. Plotch, S. J.*, et al.*, *A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription.* Cell, 1981. 23(3): p. 847-58.
21. Tatu, U.*, et al.*, *Folding and oligomerization of influenza hemagglutinin in the ER and the intermediate compartment.* EMBO J, 1995. 14(7): p. 1340-8.
22. Cros, J. F.*, et al.*, *Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses.* Virus Res, 2003. 95(1-2): p. 3-12.
23. Fujii, Y.*, et al.*, *Selective incorporation of influenza virus RNA segments into virions.* Proc Natl Acad Sci U S A, 2003. 100(4): p. 2002-7.
24. WHO. 2009; Available from: http://www.who.int/mediacentre/factsheets/fs211/en/.
25. Nguyen-Van-Tam, J. S.*, et al.*, *The epidemiology and clinical impact of pandemic influenza.* Vaccine, 2003. 21(16): p. 1762-8.
26. Monto, A. S., *Epidemiology of influenza.* Vaccine, 2008. 26 Suppl 4: p. D45-8.
27. Glezen, W. P., *Serious morbidity and mortality associated with influenza epidemics.* Epidemiol Rev, 1982. 4: p. 25-44.
28. Beveridge, W. I., *The chronicle of influenza epidemics.* Hist Philos Life Sci, 1991. 13(2): p. 223-34.
29. Pappas, G.*, et al.*, *Insights into infectious disease in the era of Hippocrates.* Int J Infect Dis, 2008. 12(4): p. 347-50.
30. Kobasa, D.*, et al.*, *Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus.* Nature, 2004. 431(7009): p. 703-7.
31. Maines, T. R.*, et al.*, *Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals.* J Virol, 2005. 79(18): p. 11788-800.
32. Shortridge, K. F.*, et al.*, *Characterization of avian H5N1 influenza viruses from poultry in Hong Kong.* Virology, 1998. 252(2): p. 331-42.
33. Garten, R. J.*, et al.*, *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. 325(5937): p. 197-201.

34. Ducatez, M. F*., et al.*, *Animal influenza epidemiology.* Vaccine, 2008. 26 Suppl 4: p. D67-9.
35. Guan, Y*., et al.*, *Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR.* Proc Natl Acad Sci U S A, 2002. 99(13): p. 8950-5.
36. Li, K. S*., et al.*, *Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia.* Nature, 2004. 430(6996): p. 209-13.
37. Molinari, N. A*., et al.*, *The annual impact of seasonal influenza in the US: measuring disease burden and costs.* Vaccine, 2007. 25(27): p. 5086-96.
38. WHO. Available from: http://www.who.int/mediacentre/factsheets/2003/fs211/en/.
39. Begley, Sharon. *Flu Pandemic Could Trigger A 'Major Global Recession'*. 2013; Available from: http://www.huffingtonpost.com/2013/01/21/flu-pandemic-recession_n_2519164.html.
40. Andrew Burns, Dominique van der Mensbrugghe, Hans Timmer. *Evaluating the Economic Consequences of Avian Influenza*. 2008; Available from: http://siteresources.worldbank.org/EXTAVIANFLU/Resources/EvaluatingAHIeconomics_2008.pdf.
41. Meltzer, M. I*., et al.*, *The economic impact of pandemic influenza in the United States: priorities for intervention.* Emerg Infect Dis, 1999. 5(5): p. 659-71.
42. Pasick, J*., et al.*, *Avian influenza: the Canadian experience.* Rev Sci Tech, 2009. 28(1): p. 349-58.
43. Stevens, J*., et al.*, *Influenza virus hemagglutinin--structural studies and their implications for the development of therapeutic approaches.* Infect Disord Drug Targets, 2007. 7(4): p. 329-35.
44. Braakman, I*., et al.*, *Folding of viral envelope glycoproteins in the endoplasmic reticulum.* Traffic, 2000. 1(7): p. 533-9.
45. Zurcher, T*., et al.*, *Mutations at palmitylation sites of the influenza virus hemagglutinin affect virus formation.* J Virol, 1994. 68(9): p. 5748-54.
46. Jin, H*., et al.*, *Palmitylation of the influenza virus hemagglutinin (H3) is not essential for virus assembly or infectivity.* J Virol, 1996. 70(3): p. 1406-14.
47. Gamblin, S. J*., et al.*, *Influenza hemagglutinin and neuraminidase membrane glycoproteins.* J Biol Chem, 2010. 285(37): p. 28403-9.
48. Sui, J*., et al.*, *Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses.* Nat Struct Mol Biol, 2009. 16(3): p. 265-73.
49. Sun, X*., et al.*, *Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism.* Cell Rep, 2013. 3(3): p. 769-78.

50. WHO. *A revision of the system of nomenclature for influenza viruses: a WHO Memorandum*. 1980; Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2395936/pdf/bullwho00427-0070.pdf.
51. Ekiert, D. C.*, et al.*, *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. 324(5924): p. 246-51.
52. Bullough, P. A.*, et al.*, *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. 371(6492): p. 37-43.
53. Daniels, R. S.*, et al.*, *Amino acid sequences of haemagglutinins of influenza viruses of the H3 subtype isolated from horses.* J Gen Virol, 1985. 66 (Pt 3): p. 457-64.
54. Potter, C. W.*, et al.*, *Determinants of immunity to influenza infection in man.* Br Med Bull, 1979. 35(1): p. 69-75.
55. Clements, M. L.*, et al.*, *Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus.* J Clin Microbiol, 1986. 24(1): p. 157-60.
56. Scherle, P. A.*, et al.*, *Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells.* J Immunol, 1992. 148(1): p. 212-7.
57. Murphy, B. R.*, et al.*, *The systemic and mucosal immune response of humans to influenza A virus.* Curr Top Microbiol Immunol, 1989. 146: p. 107-16.
58. Hu, W.*, et al.*, *Fully human broadly neutralizing monoclonal antibodies against influenza A viruses generated from the memory B cells of a 2009 pandemic H1N1 influenza vaccine recipient.* Virology, 2013. 435(2): p. 320-8.
59. Kubota-Koketsu, R.*, et al.*, *Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors.* Biochem Biophys Res Commun, 2009. 387(1):p. 180-5
60. Corti, D.*, et al.*, *Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine.* J Clin Invest, 2010. 120(5): p. 1663-73.
61. Pica, N.*, et al.*, *Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses.* Proc Natl Acad Sci U S A, 2012. 109(7): p. 2573-8.
62. Gerhard, W.*, et al.*, *Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies.* Nature, 1981. 290(5808): p. 713-7.
63. Caton, A. J.*, et al.*, *The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype).* Cell, 1982. 31(2 Pt 1): p. 417-27.
64. Breschkin, A. M.*, et al.*, *Antigenic determinants of influenza virus hemagglutinin. VIII. Topography of the antigenic regions of influenza virus hemagglutinin determined by*

competitive radioimmunoassay with monoclonal antibodies. Virology, 1981. 113(1): p. 130-40.

65. Underwood, P. A., *Mapping of antigenic changes in the haemagglutinin of Hong Kong influenza (H3N2) strains using a large panel of monoclonal antibodies.* J Gen Virol, 1982. 62 (Pt 1): p. 153-69.
66. Tsuchiya, E.*, et al.*, *Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus.* J Gen Virol, 2001. 82(Pt 10): p. 2475-84.
67. Whittle, J. R.*, et al.*, *Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin.* Proceedings of the National Academy of Sciences of the United States of America, 2011. 108(34): p. 14216-21.
68. Okuno, Y.*, et al.*, *A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains.* J Virol, 1993. 67(5): p. 2552-8.
69. Smirnov, Y. A.*, et al.*, *An epitope shared by the hemagglutinins of H1, H2, H5, and H6 subtypes of influenza A virus.* Acta Virol, 1999. 43(4): p. 237-44.
70. Smirnov, Y. A.*, et al.*, *Prevention and treatment of bronchopneumonia in mice caused by mouse-adapted variant of avian H5N2 influenza A virus using monoclonal antibody against conserved epitope in the HA stem region.* Arch Virol, 2000. 145(8): p. 1733-41.
71. Dreyfus, C.*, et al.*, *Structure of a classical broadly neutralizing stem antibody in complex with a pandemic h2 influenza virus hemagglutinin.* J Virol. 87(12): p. 7149-54.
72. Lingwood, D.*, et al.*, *Structural and genetic basis for development of broadly neutralizing influenza antibodies.* Nature. 489(7417): p. 566-70.
73. Huang, C. C.*, et al.*, *Structural basis of tyrosine sulfation and VH-gene usage in antibodies that recognize the HIV type 1 coreceptor-binding site on gp120.* Proc Natl Acad Sci U S A, 2004. 101(9): p. 2706-11.
74. Luftig, M. A.*, et al.*, *Structural basis for HIV-1 neutralization by a gp41 fusion intermediate-directed antibody.* Nat Struct Mol Biol, 2006. 13(8): p. 740-7.
75. Corti, D.*, et al.*, *A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins.* Science, 2011. 333(6044): p. 850-6.
76. Burioni, R.*, et al.*, *Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV).* Virology, 2010. 399(1): p. 144-52.
77. De Marco, D.*, et al.*, *A non-VH1-69 heterosubtypic neutralizing human monoclonal antibody protects mice against H1N1 and H5N1 viruses.* PLoS One, 2012. 7(4): p. e34415.
78. Ekiert, D. C.*, et al.*, *A Highly Conserved Neutralizing Epitope on Group 2 Influenza A Viruses.* Science, 2011. 333(6044):p. 843-50

79. Dreyfus, C., *et al.*, *Highly conserved protective epitopes on influenza B viruses.* Science, 2012. 337(6100): p. 1343-8.
80. Yoshida, R., *et al.*, *Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses.* PLoS Pathog, 2009. 5(3): p. e1000350.
81. von Itzstein, M., *The war against influenza: discovery and development of sialidase inhibitors.* Nat Rev Drug Discov, 2007. 6(12): p. 967-74.
82. De Clercq, E., *Antiviral agents active against influenza A viruses.* Nat Rev Drug Discov, 2006. 5(12): p. 1015-25.
83. Samson, M., *et al.*, *Influenza virus resistance to neuraminidase inhibitors.* Antiviral Res, 2013. 98(2): p. 174-85.
84. Das, K., *et al.*, *Structures of influenza A proteins and insights into antiviral drug targets.* Nat Struct Mol Biol, 2010. 17(5): p. 530-8.
85. Renaud, C., *et al.*, *Emerging oseltamivir resistance in seasonal and pandemic influenza A/H1N1.* J Clin Virol, 2011. 52(2): p. 70-8.
86. Thorlund, K., *et al.*, *Systematic review of influenza resistance to the neuraminidase inhibitors.* BMC Infect Dis, 2011. 11: p. 134.
87. Wang, J., *et al.*, *Discovery of spiro-piperidine inhibitors and their modulation of the dynamics of the M2 proton channel from influenza A virus.* J Am Chem Soc, 2009. 131(23): p. 8066-76.
88. Russell, R. J., *et al.*, *Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion.* Proc Natl Acad Sci U S A, 2008. 105(46): p. 17736-41.
89. Clementi, N., *et al.*, *Broad-range neutralizing anti-influenza A human monoclonal antibodies: new perspectives in therapy and prophylaxis.* New Microbiol, 2012. 35(4): p. 399-406.
90. Burton, D. R., *et al.*, *Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses.* Science, 2012. 337(6091): p. 183-6.
91. Fleishman, S. J., *et al.*, *Computational design of proteins targeting the conserved stem region of influenza hemagglutinin.* Science, 2011. 332(6031): p. 816-21.
92. Whitehead, T. A., *et al.*, *Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing.* Nat Biotechnol, 2012. 30(6): p. 543-8.
93. Pica, N., *et al.*, *Toward a universal influenza virus vaccine: prospects and challenges.* Annu Rev Med, 2013. 64: p. 189-202.
94. Palese, P., *Making better influenza virus vaccines?* Emerg Infect Dis, 2006. 12(1): p. 61-5.
95. Couch, R. B., *Seasonal inactivated influenza virus vaccines.* Vaccine, 2008. 26 Suppl 4: p. D5-9.

96. FDA, *FDA approves first seasonal influenza vaccine manufactured using cell culture technology.* 2012; Available from: http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm328982.htm.

97. FDA, *FDA approves new seasonal influenza vaccine made using novel technology.* 2013, Available from: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm335891.htm.

98. Nichol, K. L., *Efficacy and effectiveness of influenza vaccination.* Vaccine, 2008. 26 Suppl 4: p. D17-22.

99. Treanor, J. J.*, et al.*, *Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans.* Vaccine, 2001. 19(13-14): p. 1732-7.

100. FDA, *FluMist (Intranasal Influenza Virus Vaccine) Questions and Answers. Available from:* http://www.fda.gov/BiologicsBloodVaccines/Vaccines/QuestionsaboutVaccines/ucm080754.htm.

101. EMA, *Fluenz. Available from:* http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001101/human_med_001405.jsp&mid=WC0b01ac058001d125.

102. Powell, T. J.*, et al.*, *Pseudotyped influenza a virus as a vaccine for the induction of heterotypic immunity.* J Virol, 2012. 86(24): p. 13397-406.

103. Cheng, X.*, et al.*, *Evaluation of the Humoral and Cellular Immune Responses Elicited by the Live Attenuated and Inactivated Influenza Vaccines and Their Roles in Heterologous Protection in Ferrets.* J Infect Dis, 2013.

104. Belshe, R. B.*, et al.*, *The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children.* N Engl J Med, 1998. 338(20): p. 1405-12.

105. Belshe, R. B.*, et al.*, *Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age.* Vaccine, 2008. 26 Suppl 4: p. D10-6.

106. Belshe, R. B.*, et al.*, *Efficacy of live attenuated influenza vaccine in children against influenza B viruses by lineage and antigenic similarity.* Vaccine, 2010. 28(9): p. 2149-56.

107. Jang, Y. H.*, et al.*, *Immunogenicity and protective efficacy of cold-adapted X-31 live attenuated pre-pandemic H5N1 influenza vaccines.* Vaccine, 2013. 31(33):p. 3339-46

108. Baz, M.*, et al.*, *H5N1 vaccines in humans.* Virus Res, 2013.

109. Stanekova, Z.*, et al.*, *Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development.* Virol J, 2010. 7: p. 351.

110. Fiers, W.*, et al.*, *M2e-based universal influenza A vaccine.* Vaccine, 2009. 27(45): p. 6280-3.

111. El Bakkouri, K.*, et al.*, *Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection.* J Immunol, 2010. 186(2): p. 1022-31.

112. Neirynck, S.*, et al.*, *A universal influenza A vaccine based on the extracellular domain of the M2 protein.* Nat Med, 1999. 5(10): p. 1157-63.

113. Price, G. E.*, et al.*, *Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses.* PLoS One, 2010. 5(10): p. e13162.

114. Powell, T. J.*, et al.*, *Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective T-cell and antibody responses in vivo.* Vaccine, 2011. 29(3): p. 558-69.

115. Fisher, K. D.*, et al.*, *HPMA copolymers for masking and retargeting of therapeutic viruses.* Adv Drug Deliv Rev, 2010. 62(2): p. 240-5.

116. Nayak, S.*, et al.*, *Progress and prospects: immune responses to viral vectors.* Gene Ther, 2010. 17(3): p. 295-304.

117. Steel, J.*, et al.*, *Influenza virus vaccine based on the conserved hemagglutinin stalk domain.* MBio, 2010. 1(1).

118. Sagawa, H.*, et al.*, *The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region.* J Gen Virol, 1996. 77 (Pt 7): p. 1483-7.

119. Krammer, F.*, et al.*, *Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly-protective stalk-specific antibodies.* J Virol, 2013. 87(12):6542-50.

120. Wang, T. T.*, et al.*, *Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes.* Proc Natl Acad Sci U S A, 2010. 107(44): p. 18979-84.

121. Bommakanti, G.*, et al.*, *Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge.* Proc Natl Acad Sci U S A, 2010. 107(31): p. 13701-6.

122. Paschke, M., *Phage display systems and their applications.* Appl Microbiol Biotechnol, 2006. 70(1): p. 2-11.

123. Cwirla, S. E.*, et al.*, *Peptides on phage: a vast library of peptides for identifying ligands.* Proc Natl Acad Sci U S A, 1990. 87(16): p. 6378-82.

124. Fellouse, F. A.*, et al.*, *High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries.* J Mol Biol, 2007. 373(4): p. 924-40.

125. Ye, J. D.*, et al.*, *Synthetic antibodies for specific recognition and crystallization of structured RNA.* Proc Natl Acad Sci U S A, 2008. 105(1): p. 82-7.

126. Boersma, Y. L., *et al.*, *DARPins and other repeat protein scaffolds: advances in engineering and applications.* Curr Opin Biotechnol, 2011. 22(6): p. 849-57.

127. Sidhu, S. S., *et al.*, *Phage display for engineering and analyzing protein interaction interfaces.* Curr Opin Struct Biol, 2007. 17(4): p. 481-7.

128. Steiner, D., *et al.*, *Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display.* J Mol Biol, 2008. 382(5): p. 1211-27.

129. Ekiert, D. C., *et al.*, *Broadly neutralizing antibodies against influenza virus and prospects for universal therapies.* Curr Opin Virol, 2012. 2(2): p. 134-41.

130. Wang, T. T., *et al.*, *Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins.* PLoS Pathog, 2010. 6(2): p. e1000796.

131. Dintzis, H. M., *et al.*, *Molecular determinants of immunogenicity: the immunon model of immune response.* Proc Natl Acad Sci U S A, 1976. 73(10): p. 3671-3675.

132. Bachmann, M. F., *et al.*, *The influence of antigen organization on B cell responsiveness.* Science, 1993. 262(5138): p. 1448-1451.

133. Ruprecht, C. R., *et al.*, *MPER-specific antibodies induce gp120 shedding and irreversibly neutralize HIV-1.* J Exp Med, 2011. 208(3): p. 439-54.

7. Acknowledgements

I would like to thank Prof. Lars Hangartner for supervising my PhD thesis and for many discussions and advices. I also want to thank the members of my thesis committee Prof. Alexandra Trkola and Prof. Christian Münz for helpful feedback in thesis committee meetings.

I would like to express my deepest gratitude to all the members of the Institute of Medical Virology for a great working atmosphere and extensive support I received over those years. In particular I would like to thank Ines, Matteo, Marco, Donna, Nicholas, Axel and Anders.

I am very thankful to our collaborators from the Department of Biochemistry at University of Zurich (Prof. Marcus Grütter, Dr. Beat Blattman), Scripps Research Institute (Prof. Ian Wilson, Dr. Cyrille Dreyfus) for all the experiments performed, given advice and samples provided.

I am also grateful to Drs. Richard Webby and Scott Kraus from the St. Jude Children's Research Hospital, Memphis, TN, USA, Yves Thomas and Laurent Kaiser from University Hospital of Geneva, Switzerland, and Rodney Daniels from the National Institute of Medical Research, London, UK, for kindly providing viruses form their repository.

Most of all I am very grateful to all my friends and parents for believing in me for all those years.

8. Curriculum vitae

Surname:	WYRZUCKI
First names:	Arkadiusz Maciej
Date of birth:	07.06.1985
Nationality:	Polish

Education:

09.2009- 08.2013	University of Zurich (Switzerland), Ph.D. in molecular virology
07.2008-06.2009	University of Chicago (USA), M.Sc. in molecular biology
09.2005-06.2007	University of Gdansk-Medical University of Gdansk (Poland): B.Sc. in biotechnology
09.2001-06.2004	Jan and Jedrzej Sniadecki VI Highschool in Bydgoszcz (Poland)

Employed as PhD student at the University of Zurich since 09.2009.

Diploma subject: human antibodies and recombinant vaccines.

Title of diploma thesis: Characterization of Influenza A-neutralizing, Heterosubtypic Antibodies Isolated with a Newly Developed Antigen.